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MOLECULAR CHARACTERIZATION OF VIBRIO CHOLERAE GENES
FLGO AND FLGP

by

DAVID C. MORRIS, B.S.

THESIS

Presented to the Graduate Faculty of
The University of Texas at San Antonio
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College of Science
Department of Biology
December 2006

Dedication

To my Dad who provided me the opportunities to succeed and the support to pursue my dreams. In memory of my Mom whose presence and encouragement are ever felt in all my endeavors. And to my wife for her love, support, and encouragement in all of my ambitions; and for putting up with me through them all.

The views expressed in this article are those of the author and do not reflect the official policy or position of the United States Air Force, Department of Defense, or the U.S. Government.

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December 2006

MOLECULAR CHARACTERIZATION OF VIBRIO CHOLERAE GENES FLGO AND FLGP

David Christopher Morris, MS
The University of Texas at San Antonio, 2006

Supervising Professor: Karl E. Klose, PhD

Vibrio cholerae, a human pathogen and causative agent of the human diarrheal disease cholera, is a highly motile bacterium by virtue of a single, sheathed, polar flagellum. Motility has been inferred to play an important role in virulence and two genes were previously identified by our lab that appeared to be regulated by the flagellar regulatory protein FlrC, VC2207 and the gene immediately downstream VC2206 (annotated as *flgO* and *flgP*, respectively). In an effort to determine the roles of FlgO and FlgP, in frame chromosomal deletions were constructed in the coding sequences of *flgO* and *flgP* of *V. cholerae*. A deletion removing the entire coding sequence for FlgO ($\Delta flgO$) was constructed in wild-type (KKV598) and the same done for FlgP ($\Delta flgP$). Our results demonstrate that FlgO is a secreted protein that plays a role in transcription of Class IV flagellins, is required for a motile phenotype and does not play a significant role in colonization of the infant mouse small intestine. FlgP is an outer membrane lipoprotein that is required for a motile phenotype, and plays a significant role in colonization of the infant mouse small intestine.

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GENERAL INTRODUCTION TO *VIBRIO CHOLERAE* AND BACKGROUND

Vibrio cholerae is a human pathogen and the causative agent of the human diarrheal disease cholera. The modern history of cholera began with the first recorded pandemic of cholera in 1817 and since there have been seven distinct pandemics, the last of which is currently still active (Pollitzer, 1959; Kaper *et. al.*, 1995). The bacterium that causes cholera was not identified until 1854 by an Italian physician named Pacini, but his ideas on the organism were contradicted by an influential Italian physician Maurizio Bufalini and Pacini's data was ignored by the scientific community. Robert Koch rediscovered cholera in 1884 and named the organism *Vibrio comma*. Later Pacini's work was recognized and the name was changed back to *Vibrio cholerae* (Bentivoglio and Pacini, 1995; Pollitzer, 1959). *V. cholerae* is a straight or curved gram-negative rod that is motile by a single, polar, sheathed flagellum. *V. cholerae* is in the genus *Vibrio* and the Family *Vibrionaceae* (Kay *et. al.*, 1994). Approximately 200 serotypes of *V. cholerae* have been described, while pandemics have only been associated with the O-1 and O-139 serogroups; the non-O1 and non-O139 serogroups only occasionally cause sporadic cases of diarrheal illness (Lipp *et. al.*, 2002; Kay *et. al.*, 1994). There are three specific types of *V. cholerae* that are known to cause pandemic cholera and these are *V. cholerae* O-1 classical biotype, *V. cholerae* O-1 El Tor biotype, and *V. cholerae* O-139 (Blake, 1994). *V. cholerae* O-1 classical biotype is known to have caused the fifth and sixth pandemics, and *V. cholerae* O-1 El Tor biotype, after causing only sporadic cases before 1961, was the organism that caused the seventh pandemic which continues today. In 1992, *V. cholerae* O-139 was identified as the agent causing epidemic disease in populations where *V. cholerae* O-1 was endemic in India and Bangladesh.

Cholera is a disease that affects communities that are overcrowded, with poor sanitation and water supplies that are unsafe for drinking. Outbreaks can be sporadic occurring anywhere in the world where sanitation, water supplies, food safety and hygiene practices are inadequate. Cholera is an acute intestinal infection that is characterized by the sudden onset of painless watery diarrhea, nausea and vomiting. The mortality rate for individuals with severe untreated cholera is up to 50% while appropriate treatment in a timely manner reduces that rate to less than 1%. The World Health Organization (WHO) reported that in the year 2000 some 140,000 cases of cholera were reported resulting in approximately 5,000 deaths in Africa, Latin America, Asia, Europe and Oceania (WHO, 2001; WHO, 2000).

Pathogenesis

V. cholerae is acquired by the consumption of food or water that has been contaminated. Once inside the body, the bacteria must pass through the acid barrier of the stomach before reaching the mucus lining of the small intestine. The bacteria then must penetrate the mucus lining of the small intestine and adhere to the epithelial cells of the intestine to cause infection (Prouty and Klose, 2006). Once the bacteria adhere to the intestinal epithelial cells, they begin to express several critical virulence factors including cholera toxin (CT) and toxin co-regulated pilus (TCP). The CT produced by *V. cholerae* is one of the most critical virulence factors associated with *V. cholerae*. CT is the cause of the copious amounts of watery diarrhea characteristic of a cholera infection. CT is an ADP-ribosylating toxin that leads to an increase in adenylate cyclase activity which increases the amount of cyclic AMP in the host cells. The increase in cyclic AMP causes increased secretion of Cl^- ions and CT also blocks the normal function of the intestinal mucosa to absorb the Cl^- ions leading to an osmotic imbalance. The osmotic imbalance causes large amounts of water to flow into the intestinal lumen resulting in

the copious amounts of watery diarrhea (Prouty and Klose, 2006). Untreated, the large volumes of diarrhea can lead to dehydration and potentially death. TCP is considered to be the most important colonization factor, it has two key roles in pathogenicity; the first is that it is required to colonize the host. It is a key adherence factor for *V. cholerae* (Reidl and Klose, 2002). TCP also serves as the receptor for CTX ϕ , a filamentous bacteriophage that carries the structural genes for CT (Waldor and Mekalanos, 1996).

The virulence factors CT and TCP are controlled by a regulatory cascade referred to as the ToxR regulon; the regulatory factors within the ToxR regulon continue to grow as ongoing research uncovers new elements within the system (Prouty and Klose, 2006; Di Rita, 1992). ToxT is a member of the ToxR regulon that is transcriptionally controlled by ToxR. ToxT has been shown to directly activate expression of virulence factors including CT and TCP within the ToxR regulon (Otteman and Mekalanos, 1994; DiRita *et. al.*, 1991).

There are several additional virulence factors associated with *V. cholerae* including lipopolysaccharide (LPS), motility and chemotaxis, porins, RTX toxin, hemagglutinin/protease, HlyA hemolysin and a type II secretion system (Prouty and Klose, 2006). Below I will focus on motility and the role it plays in virulence of *V. cholerae*.

Motility and Pathogenesis

The role of motility in pathogenesis is a complex issue that involves aspects of flagellar synthesis, chemotaxis, virulence factor production and motility itself. And even though motility has been linked to *V. cholerae* pathogenesis; the exact role it plays is still under investigation. Guentzel and Berry (1975) showed that non-motile strains of Ogawa and Inaba serotypes of classical *V. cholerae* and El Tor Biotype cholera showed a marked reduction in virulence when compared to motile strains. Their study suggested that motility may contribute to virulence by

increasing the vibrios chance for association with the intestinal mucosa (Guentzel and Berry, 1975). It has also been shown that non-motile mutants are unable to attach to isolated rabbit intestinal brush border membranes *in vitro* (Freter and O'Brien, 1981); and in the rabbit ileal loop model non-motile mutants cause less fluid accumulation and in the removable intestinal tie adult rabbit diarrhea (RITARD) model non-motile mutants cause less disease (Richardson, 1991). The results for non-motile mutants in different animal models has provided conflicting data regarding virulence, yet in human volunteer studies data shows that motility may play a role in virulence (Reidl and Klose, 2002).

In a study conducted by Coster *et. al.*, (1995), attenuated vaccine strains of *V. cholerae* O-139 were made by deleting several copies of the CT element from M010 and A14456 and then inserting a construct of the B subunit of CT generating Bengal-3 and VRI-16. A stable non-motile mutant was derived from Bengal-3 and designated Bengal-15. The two non-motile strains, the Bengal-3 strain and the parent M010 were all tested in human volunteers and both motile strains caused cholera disease while the two non-motile strains did not produce significant symptoms. One non-motile strain, Bengal-15, exhibited immunogenicity and was identified as a potential live attenuated vaccine candidate (Coster *et. al.*, 1995).

Gardel and Mekalanos (1996) looked at non-motile mutants and hyperswarming mutants to see how different motility phenotypes affected virulence factor expression. They classified the motility mutant phenotypes into three classes of hyperswarmer mutants and the non-motile mutants. Two of the three hyperswarmer mutants were found to be defective for autoagglutination which is associated with TCP expression, a ToxR regulated colonization factor; however, in contrast to those findings the non-motile mutants were all found to exhibit autoagglutination even under conditions that typically inhibit the phenotype. All of the non-motile mutants examined in the study expressed increased levels of TCP pilin, CT and cell-

associated hemolysin and in the infant mouse model had little defect for intestinal colonization. An inverse relationship between motility and virulence gene expression was shown; expression of one may inhibit expression of the other (Gardel and Mekalanos, 1996). Nidia et. al (2000) proposed a potential coupling between virulence factor production and motility through the Na⁺ gradient across the membrane that drives *V. cholerae* flagellar rotation and the Na⁺ gradient's regulation of ToxR and TcpP dependent transcription of the *toxT* gene.

Flagellar synthesis and regulation also has been studied providing insights into the effects specific genes and regulatory elements have on pathogenesis. Prouty *et. al.*, (2001) proposed a novel flagellar gene transcriptional hierarchy for *V. cholerae* unique from both *C. crescentus* and *S. typhimurium* that was composed of four classes of genes. Class I contains only one gene: the σ^{54} -dependent activator FlrA. Class II genes are σ^{54} and FlrA dependent and encode components of the MS ring, switch, export apparatus and the regulatory factors FlrB/FlrC, FlgM and σ^{28} . Class III genes are σ^{54} and FlrC dependent and encode the hook and basal body, the hook-filament adaptors, sodium motor protein and the core flagellin FlaA. The final class is the Class IV genes; these are σ^{28} dependent and encode other flagellins, other sodium motor proteins, the filament cap, chaperones and some chemotaxis proteins.

Research into the Class III genes and the Class III regulator FlrC has produced interesting results on motility and pathogenesis. The flagellum of *V. cholerae* is composed of at least five flagellins, but only the protein encoded by the Class III gene *flaA* is critical for motility and *flaA* requires the regulatory proteins σ^{54} and FlrB/FlrC for expression (Klose and Mekalanos, 1998a; Klose and Mekalanos 1998b). Correa *et. al.*, (2000) showed that phosphorylation of *V. cholerae* FlrC is required for *flaA* transcription, flagellar synthesis, motility and enhanced colonization in the infant mouse animal model. These findings support the idea that there may be a connection between flagellar synthesis/regulation, motility and pathogenesis. Previously collected

microarray data has shown that FlgO and FlgP in *V. cholerae* appear to be up-regulated by the Class III regulator FlrC and may play a role in motility and pathogenesis.

Objectives

Elucidation of the roles of the genes involved in flagellar synthesis and regulation is an ongoing process to understand how *V. cholerae* assembles a flagella and the effects on virulence to determine if the genes are potential targets for vaccine development. FlgO and FlgP lie in the same region of the genome as a large number of genes that control flagellar synthesis or form components of the flagella. Characterizing these genes as flagellar genes and understanding the role of these proteins in flagellar synthesis and colonization will help us to better understand the components of flagellar synthesis and identify potential vaccine targets. The main research objectives are:

(i) Characterization of FlgO and FlgP proteins and their role in motility and virulence.

The results of the research for this thesis show that in frame deletions of the *flgO* and *flgP* genes in *V. cholerae* generate mutants that are non-motile in swarm agar. Characterization of FlgO showed that the protein appears to be secreted to the inner membrane or periplasmic space, it positively effects transcription of Class IV flagellar genes, and deletion prevents secretion of FlgM and causes the mutant bacteria to produce atypical flagella. Characterization of FlgP indicated that it is a lipoprotein that localizes to the outer membrane; deletion causes a 10-fold decrease in colonization of the infant mouse small intestine, prevents secretion of FlgM and causes production of an atypical flagella.

MATERIALS AND METHODS

Media

Luria Broth (LB) was used for both liquid media and agar plates. The LB was routinely supplemented with antibiotics as required (Ampicillin at 50µg/mL, Streptomycin at 100µg/mL and to produce recombination events in *V. cholerae* Streptomycin at 250mg/mL). When using the pBAD vector, LB was supplemented with 0.1% arabinose. Motility of bacterial strains was measured in 0.3% LB agar supplemented with appropriate antibiotics and 0.1% arabinose. Confirmation of *V. cholerae* strains was performed using Thiosulfate Citrate Bile Sucrose (T.C.B.S.) Agar. LB was also supplemented with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) when using pUC118 plasmid during initial cloning events into the multiple cloning site (MCS) and for bacterial growth from animal studies.

Plasmid Construction

A complete list of plasmids and oligonucleotide primers used in this study can be found in Table 1 and Table 2 respectively. Restriction sites used in cloning are underlined in the oligonucleotides listed in Table 2. The plasmid pKEK782 was constructed by using PCR to amplify the *flgO* promoter from *V. cholerae* O395 with oligonucleotides 2208p Down and 2208p Up. The PCR fragment was digested with *Bam*HI and *Eco*RI and ligated into pRS551 digested with the same enzymes.

The plasmid pKEK 802 was constructed by using two-step PCR to amplify a region upstream and downstream of *flgP* and then the two products were PCR amplified into a single fragment that deleted *flgP*. First the upstream segment of Δ *flgP* was amplified from KKV598 using oligonucleotides 2206 Down Bgl2 and 2206 Δ Up. Then the downstream portion of Δ *flgP*

TABLE 1. Complete list of plasmids used in this study.

Plasmids	Genotype	Source/Reference
pKAS32	pGP704, <i>rpsL</i> , Amp ^r	Skorupski and Taylor (1996)
pUC118	Phagemid cloning vector, Amp ^r	Vieira and Messing (1982)
pWKS30	pSC101 replicon; Amp ^r ; MCS(-)	Wang and Kushner (1991)
pBAD24	ColE1 <i>ori</i> Amp ^r <i>araBAD</i> promoter	Guzman <i>et. al.</i> , (1995)
pRS551	Transcriptional <i>lacZ</i> fusion vector, Amp ^r Kan ^r	Simons <i>et. al.</i> , (1987)
pKEK76	<i>flaC</i> promoter- <i>lacZ</i> fusion in pRS551	Klose and Mekalanos (1998)
pKEK77	<i>flaD</i> promoter- <i>lacZ</i> fusion in pRS551	Klose and Mekalanos (1998)
pKEK79	<i>flaB</i> promoter- <i>lacZ</i> fusion in pRS551	Klose and Mekalanos (1998)
pKEK80	<i>flaA</i> promoter- <i>lacZ</i> fusion in pRS551	Klose and Mekalanos (1998)
pKEK81	<i>flaE</i> promoter- <i>lacZ</i> fusion in pRS551	Klose and Mekalanos (1998)
pKEK331	<i>flgK</i> promoter- <i>lacZ</i> fusion in pRS551	Prouty <i>et. al.</i> , (2001)
pKEK332	<i>flgB</i> promoter- <i>lacZ</i> fusion in pRS551	Prouty <i>et. al.</i> , (2001)
pKEK415	<i>flaG</i> promoter- <i>lacZ</i> fusion in pRS551	Prouty <i>et. al.</i> , (2001)
pKEK474	FLAG- <i>flgM</i> in pBAD24	Correa <i>et. al.</i> , (2004)
pKEK782	<i>flgO</i> promoter- <i>lacZ</i> fusion in pRS551	This study (Fen Peng)
pKEK802	$\Delta flgP$ in pKAS32	This study (Fen Peng)
pKEK845	<i>flgP</i> in pBAD24	This study (Fen Peng)
pKEK846	<i>flgO</i> in pBAD24	This study (Fen Peng)
pKEK874	$\Delta flgO$ A+B fragment in pUC118	This study
pKEK918	$\Delta flgO$ C'+D fragment in pUC118	This study
pKEK920	$\Delta flgO$ in pKAS32	This study
pKEK944	<i>flgP</i> -FLAG tag in pBAD24	This study
pKEK945	<i>flgO</i> -FLAG tag in pBAD24	This study
pKEK978	Natural promoter <i>flgO</i> in pWSK30	This study (Fen Peng)
pKEK1033	<i>flgP</i> without stop codon in pBAD24	This study
pKEK1034	<i>flgO</i> without stop codon in pBAD24	This study
pKEK1035	<i>flgP-phoA</i> fusion in pBAD24	This study
pKEK1036	<i>flgO-phoA</i> fusion in pBAD24	This study
pKEK1037	<i>flgP</i> -C18G in pBAD24	This study
pKEK1038	<i>flgP</i> -FLAG tag C18G in pBAD24	This study
pKEK1044	Natural promoter $\Delta flgO$ + <i>flgP</i> in pWSK30	This study (Fen Peng)
pKEK1079	CJ1026 in pBAD24	This study
pKEK1080	Natural promoter $\Delta flgO$ + <i>flgP</i> C18G in pWSK30	This study
pKEK1081	<i>flgO-flgP</i> in pBAD24	This study

TABLE 2. Complete list of oligonucleotides used in this study. Restriction sites have been underlined.

Oligonucleotides	Sequence
2208p Down	5'-GCGGATCCCAATTGGTTATAAAATCGGGGCGTATGC-3'
2208p Up	5'-GCGAATTCAGATCTGTGTAAGCGGGCGACCTCTTC-3'
2206 Down Bgl2	5'-GCGAGATCTGAGCATGACGGAAGATTTGATG-3'
2206 ΔUp	5'-TTTGTCTGTTTTAGTAAGCTAGATTTTCATCATC-3'
2206 ΔDown	5'-TAGCTTACTAAAACGACAAATGACCCTATTC-3'
2206 Up EcoRI	5'-GCGAATTCGAAAAATGCGCAAGAACTCGTG-3'
2207 Down BHI	5'-GCGGATCCGTATTGCTCAACGTGGCAAGCCGTTG-3'
Δ2207 Up RI	5'-GCGAATTCAGATATACTATGGCGCGCATTTCAG-3'
Δ2207 Down MfeI	5'-GCGCCAATTGACCGTGATTTTACGTCCATAGGAG-3'
2207 Up RI	5'-GCGAATTCAAAAGCGGCAATCATTGCCGCTTTTG-3'
PB	5'-AAATCACGGTCAGATATACTATGGCGCGCATTTC-3'
PC	5'-AGTATATCTGACCGTGATTTTACGTCCATAGGAG-3'
2206 Flag Tag Up	5'-GCGTCTAGACTACTTGTCTGTCGTCATCCTTGTAGTCCTCGAGG AATAGGGTCATTTGTCTGTTTTTCGG-3'
2207 Flag Tag Up	5'-GCGTCTAGACTACTTGTCTGTCGTCATCCTTGTAGTCCTCGAGT GGACGTAAAATCACGGTGTACGGCTG-3'
VC2206 Down NcoI	5'-CATGCCATGGCTATGAAATCTAGCTTACTACTC-3'
ΔVC2206 Up XbaI	5'-TGCTCTAGACTAGAAATAGGGTCATTTGTCTG-3'
VC2206 Up XmaI w/o Stop	5'-TGCCCCGGGGAATAGGGTCATTTGTCTG-3'
VC2207 Down NcoI	5'-CATGCCATGGCCCCGCGCCATAGTATATCTGGAG-3'
ΔVC2207 Up XbaI	5'-TGCTCTAGACTATGGACGTAAAATCACGGT-3'
VC2207 Up XmaI w/o Stop	5'-TGCCCCGGGTGGACGTAAAATCACGGTGTAC-3'
phoA Down XmaI	5'-TGCCCCGGGCCCCGGACACCAGAA-3'
phoA Up HindIII	5'-TGCAAGCTTTCATCTAGATTTTCAGCCCCAG-3'
Fwd Cys to Gly – VC2206	5'-CTTAATGATGACGGGAGGCCAACCCCTTGCAAAGC-3'
Rv Cys to Gly – VC2206	5'-GCTTTGCAAGGGTTGGCCTCCCGTCATCATTAAAG-3'

was amplified from KKV598 using oligonucleotides 2206 ΔDown and 2206 Up EcoRI. Then using the two fragments of *ΔflgP*, one final PCR reaction using the oligonucleotides 2206 Down Bgl2 and 2206 Up EcoRI was performed to create the full length *ΔflgP* fragment, which was digested with *BglII* and *EcoRI* and ligated into pKAS32 (Skorupski and Taylor, 1996) digested with the same enzymes creating pKEK802.

Inducible plasmids containing *flgO* and *flgP* were created to complement the in-frame deletions of the genes. The plasmid pKEK845 was created with the full length *flgP* gene by PCR amplifying *flgP* from KKV598 using the oligonucleotides VC2206 Down NcoI and VC2206 Up XbaI. The PCR fragment was then digested with *NcoI* and *XbaI* and ligated into pBAD24 (Guzman *et. al.*, 1995) digested with the same enzymes. The *flgO* gene was amplified from KKV598 using the oligonucleotides VC2207 Down NcoI and VC2207 Up XbaI and the resulting fragment was digested with *NcoI* and *XbaI* and ligated into pBAD24 digested with the same enzymes creating pKEK846.

The plasmid used to create the *flgO* deletion in KKV598 was generated by first creating plasmids that contained the 5' portion of the deletion and the 3' portion of the deletion. Plasmid pKEK874 was created by PCR amplifying the 5' portion of *flgO* from KKV598 using the oligonucleotides 2207 Down BHI and Δ2207 Up RI. The product was digested with *BamHI* and *EcoRI* and ligated into pUC118 (Vieira and Messing, 1982) that had been similarly digested, forming the 5' fragment of the *ΔflgO* deletion. Plasmid pKEK918 was created by PCR amplifying the 3' portion of *flgO* from KKV598 using the oligonucleotides Δ2207 Down MfeI and 2207 Up RI. The product was digested with *MfeI* and *EcoRI* and ligated into pUC118 that had been digested with *EcoRI* and treated with calf intestinal phosphatase (CIP), forming the 3' portion of the *ΔflgO* deletion. To create pKEK920 the 5' fragment of the *ΔflgO* deletion was PCR amplified from pKEK 874 using the oligonucleotides 2207 Down BHI and Δ2207 Up RI

and the 3' fragment was PCR amplified using the oligonucleotides Δ 2207 Down MfeI and 2207 Up RI. The PCR products were the PCR amplified using the oligonucleotides PC and PB to form the Δ *flgO* PCR fragment. The Δ *flgO* PCR fragment was digested with *Bam*HI and *Eco*RI and then ligated into pKAS32 which was similarly digested. This plasmid was used to create the in frame *flgO* deletion in *Vibrio cholerae* strain KKV598 giving rise to KKV2096.

To make visualization of the FlgO and FlgP proteins possible in Western blots without specific antibodies against each protein, FLAG-tagged versions of each were constructed and inserted into arabinose inducible vectors. The *flgO*-FLAG-tagged plasmid was created by PCR amplifying the *flgO* gene from pKEK846 using the oligonucleotides VC2207 Down NcoI and 2207 Flag Tag Up, putting the FLAG-tag at the C-terminus. The PCR fragment and pBAD24 were then digested with *Nco*I and *Xba*I and ligated together forming plasmid pKEK945. The *flgP*-FLAG-tagged version was created by PCR amplifying the *flgP* gene from pKEK845 using the oligonucleotides VC2206 Down NcoI and 2206 Flag Tag Up, putting the FLAG-tag at the C-terminus. The PCR fragment and pBAD24 were then digested with *Nco*I and *Xba*I and ligated together forming plasmid pKEK944.

The plasmid pKEK978 was constructed containing the *flgO* gene with its natural promoter in pWSK30 (Wang and Kushner, 1991). pKEK978 was constructed by PCR amplifying *flgO* from wild-type (KKV598) genomic DNA using the oligonucleotides VC2208p Up and VC2207 Up XbaI. The PCR fragment was digested with *Eco*RI and *Xba*I, and then ligated into pWSK30 digested with the same enzymes.

Plasmids containing *flgO* and *flgP* fused with *phoA* were made in a stepwise process. To construct the plasmid containing *flgO-phoA* fusion, first the plasmid pKEK1034 was created by PCR amplifying pKEK846 with the oligonucleotides VC2207 Down NcoI and VC2207 Up XmaI w/o Stop. The PCR fragment was digested with *Nco*I and *Xma*I and ligated into pBAD24

digested with the same enzymes. Next *phoA* was PCR amplified from *E. coli* using oligonucleotides *phoA* Down *XmaI* and *phoA* Up *HindIII*. The PCR fragment was digested with *XmaI* and *HindIII* and ligated into pKEK1034 digested with the same enzymes generating the *flgO-phoA* fusion in pBAD24 designated pKEK1036. The plasmid containing the *flgP-phoA* fusion was constructed in a similar manner as the *flgO-phoA* fusion. First, *flgP* without the stop codon was amplified from pKEK845 using oligonucleotides VC2206 Down *NcoI* and VC2206 Up *XmaI* w/o Stop. The PCR fragment was digested with *NcoI* and *XmaI* and ligated into pBAD24 digested with the same enzymes forming pKEK1033. Next *phoA* was PCR amplified from *E. coli* using oligonucleotides *phoA* Down *XmaI* and *phoA* Up *HindIII*. The PCR fragment was digested with *XmaI* and *HindIII* and ligated into pKEK1033 digested with the same enzymes generating the *flgO-phoA* fusion in pBAD24 designated pKEK1035.

Three plasmids were made that contained a cysteine to glycine mutation at amino acid eighteen (C18G) in *flgP*. All three plasmids were made using the same oligonucleotides: Fwd Cys to Gly – VC2206 and Rv Cys to Gly – VC2206. The oligonucleotides were amplified with the plasmids pKEK845, pKEK944 and pKEK1044 and the PCR products were digested with *DpnI* to remove the original plasmid DNA generating pKEK1037, pKEK1038 and pKEK1080 respectively. The plasmid pKEK1044 was constructed by amplifying the $\Delta flgO$ fragment from the $\Delta flgO$ mutant chromosomal DNA (KKV2096) using oligonucleotides VC2208p Up and VC2206 Up *XbaI*. The PCR fragment was digested with *EcoRI* and *XbaI* then ligated into pWSK30 that was digested with the same enzymes.

An inducible plasmid containing a homolog of FlgP from *Campylobacter jejuni* was created. The plasmid pKEK1079 was created using the oligonucleotides CJ1026 Down *NcoI* and CJ1026 Up *XbaI* for PCR with genomic *Campylobacter jejuni* 81-176 DNA. The PCR

fragment was digested with *NcoI* and *HindIII* and ligated into pBAD24 digested with the same enzymes.

An inducible plasmid containing both the *flgO* and *flgP* gene was constructed using the oligonucleotides VC2207 Down *NcoI* and ΔVC2206 Up *XbaI*. PCR was performed using KKV598 to isolate the fragment and the fragment was then digested with *NcoI* and *XbaI*. Then the fragment was ligated into pBAD24 digested with the same enzymes creating pKEK1081.

Bacterial Strains

Table 3 contains a complete list of bacterial strains used in this study. *Escherichia coli* strain DH5α (Hanahan, 1983) was used for all cloning experiments, while a *dapA* strain (gift of Rob Edwards, San Diego State University) was used for all cloning experiments. The *ΔtoxT acfA-phoA V. cholerae* strain KKV2095 was constructed by CPT1ts phage transduction (Hava and Camilli, 2001) of *acfA-phoA* from KP9.62 (Peterson and Mekalanos, 1988) into VJ740 (Champion *et. al.*, 1997). The *ΔflgP- ΔlacZ V. cholerae* strain KKV1965 was constructed using pKEK802 (*ΔflgP* in pKAS32) to recombine *ΔflgP* onto the chromosome of *V. cholerae* strain KKV598. The *ΔflgO-ΔlacZ V. cholerae* strain KKV2096 was constructed using pKEK920 (*ΔflgO* in pKAS32) to recombine *ΔflgO* onto the chromosome of *V. cholerae* strain KKV598. Allelic exchange was confirmed by PCR and/or sequencing.

β-galactosidase Assay

V. cholerae strains were transformed with plasmids containing promoter-*lacZ* fusions listed in Table 1, grown in LB supplemented with appropriate antibiotics and 0.1% arabinose at 37°C. The samples were assayed at an optical density of 600nm of approximately 0.4 to 0.8, then

TABLE 3. Complete list of bacterial strains used in this study.

Strains	Genotype	Source/Reference
<i>V. cholerae</i>		
KKV56	O395 $\Delta rpoN1$, $\Delta lacZ$	Klose and Mekalanos (1998b)
KKV59	O395 $\Delta flrA1$, $\Delta lacZ$	Klose and Mekalanos (1998b)
KKV61	O395 $\Delta toxR$	Klose and Mekalanos (1998b)
KKV98	O395 $\Delta flrC1$, $\Delta lacZ$	Klose and Mekalanos (1998b)
KKV598	O395 $\Delta lacZ$	Correa <i>et. al.</i> , (2000)
KKV1113	O395 $\Delta fliA1$, $\Delta lacZ$	Prouty <i>et. al.</i> , (2001)
KKV1247	O395 $\Delta fliF$, $\Delta lacZ$	Correa <i>et. al.</i> , (2004)
KKV1965	O395 $\Delta flgP$, $\Delta lacZ$	This study (Fen Peng)
KKV2095	O395 $\Delta toxT$ <i>acfA-phoA</i>	This study (Fen Peng)
KKV2096	O395 $\Delta flgO$, $\Delta lacZ$	This study
<i>E. coli</i>		
DH5 α	F ⁻ <i>deoR endA1 hsdR17 supE44 thi-1</i> <i>recA1 gyrA96 relA1</i> $\Delta(argF-lacZYA)$	Hanahan (1983)
<i>dapA</i>	U196 ($\phi 80dlacZ\Delta M15$)	(gift of Rob Edwards, San Diego State University)

permeabilized with chloroform and sodium dodecyl sulfate (SDS), and assayed for β -galactosidase activity by the method described by Miller (1992).

Alkaline Phosphatase Assay

V. cholerae strains were transformed with plasmids pKEK1036 and pKEK1035, that encoded wild-type *flgO* and *flgP* proteins (respectively) without the stop codon fused to *phoA* which was deleted of its signal sequence, then grown in LB supplemented with appropriate antibiotics and 0.1% arabinose at 37°C. The samples were assayed at an optical density (600nm) of approximately 0.4 to 0.7, then permeabilized with chloroform and sodium dodecyl sulfate (SDS), and assayed for alkaline phosphatase activity by the method described by Michaelis (1983).

Protein Detection

Isolation of *V. cholerae* outer membrane (OM) proteins was performed using a method adapted from Lohia *et. al.*, (1984). Strains were grown overnight in LB supplemented with appropriate antibiotics and 0.1% arabinose. Strains were normalized to an OD₆₀₀ of 2.0 and 1mL of culture was spun down. The pellet was resuspended in 4M urea, incubated 10 minutes, then pelleted at 9,000 revolutions per minute (rpm), and the pellet discarded. The supernatant was then spun at 11,000 rpm and the pellet discarded. The supernatant was transferred to a Sorvall 10mL polyallomer thin-walled ultracentrifuge tube and the remainder of the volume was filled with 4M urea and spun in a Sorvall Discovery 90SE ultracentrifuge using the Sorvall TH641 rotor at 40,000 rpm at 4°C for 1 hour. Supernatant was removed and pellet was resuspended in 100 μ L 10mM HEPES with 10 μ g DNase and 10 μ g RNase and incubated for 30 minutes. Then

10 mL 4 M urea was added and centrifuged at 40,000 rpm, 4°C for 1 hour. The supernatant was removed and the pellet resuspended in 1X sample buffer.

Detection of FLAG-FlgM secretion was accomplished by transforming pKEK474 into KKV598 (wild type), KKV1247 (Δ *fliF*), KKV1965 (Δ *flgP*) and KKV2096 (Δ *flgO*). The strains were grown in LB plus 0.1% arabinose at 37°C for 2 hours. Samples were removed and centrifuged at 12,500 rpm for 30 min. The samples were concentrated with 10% trichloroacetic acid (TCA). Samples were then centrifuged in a Sorvall Discovery 90SE ultracentrifuge using the Sorvall TH641 rotor at 20,000 rpm for 1.5 hours and the pellets were washed twice with ice cold acetone.

Samples were separated by SDS-15% polyacrylamide gel electrophoresis, probed by Western immunoblotting using a Bio-Rad transblotter with anti-FLAG M2 (Sigma) monoclonal antibody, and detected with ECL detection reagent (Amersham-Pharmacia). SDS-15% polyacrylamide gels were also stained with Coomassie® Brilliant Blue G-250 (Bio-Rad) for quantitation and visualization of the protein.

CT assays were performed on culture supernatants using GM₁-enzyme linked immunosorbent assay (ELISA) with rabbit polyclonal antiserum against the purified B subunit of CT. ELISA plates were prepared by incubating 200µL of a solution containing 10µg/mL of GM₁ gangliosides in 60mM sodium carbonate at 37°C for 4 hours. The plate was then washed three times with 1X phosphate buffered saline (PBS) and incubated again in 200µL 1X PBS with 2mg/mL of bovine serum albumin (BSA) at 37°C for 4 hours. The solution was then discarded, the plate washed three times with 1X PBS, sealed, and stored at 4°C until used. Samples were prepared by growing the bacteria under inducing conditions with appropriate antibiotics and arabinose. The cells were quantitated by OD₆₀₀ measurement and then 1mL of each sample was centrifuged at high speed for five minutes. The ELISA plate was then prepared for the assay by

adding 200 μ L of 1X PBS with 2mg/mL BSA to all wells except the first row. 200 μ L of cell free supernatant from each sample was added to the first row including a positive and negative control. A serial dilution of 1:3 was then made in the remainder of the rows. The ELISA plate was then incubated at 37°C for 30 minutes, and washed with 1X PBS with 2mg/mL BSA. Next 200 μ L 1X PBS with 2mg/mL BSA containing a 1:2000 dilution of anti-cholera toxin antibody was added and incubated for 30 minutes at 37°C, then washed with 1X PBS with 2mg/mL BSA. 200 μ L of a 1:4000 dilution of alkaline-phosphatase conjugated anti-mouse antibody in 1X PBS with 2mg/mL BSA was added, incubated for 30 minutes at 37°C, then washed with 1X PBS with 2mg/mL BSA. Development was accomplished by adding 200 μ L of 1M Tris pH8.0 containing 2mg/mL p-nitrophenylphosphate. Development was stopped using 50 μ L 0.1M K₂HPO₄, absorbance measured at 405nm using Bio-Rad's Model 680 Microplate Reader, and analysis was performed using the software Microplate Manager version 5.2.1 from Bio-Rad. This protocol was adopted from Ristaino *et. al.*, (1983).

TCP was measured indirectly by measurement of TcpA expression by CTX ϕ -Kan phage transduction, as previously described by Waldor and Mekalanos (1996). The CTX ϕ -Kan strain was prepared by growing overnight at 37°C in 5mL LB with 50 μ g/mL Kanamycin. The culture was centrifuged at 12,000 X g for 5 minutes to pellet the cells. The cell free supernatant was filter sterilized using a 0.2 μ m filter and stored at 4°C until needed.

The phage transduction assay was performed by growing bacterial strains to be tested in 5mL LB, under inducing conditions, overnight with appropriate antibiotics and arabinose if required. The cells were quantitated by OD₆₀₀ measurement and then 100 μ L of each sample, in triplicate, was added to 1.5mL eppendorf tubes. One set of tubes containing 100 μ L LB only was set-up to ensure there was no bacterial contamination in the phage stock. For each strain and the

control 1, 10 and 100 μ L of phage stock was added to one concentration per tube of the triplicate tubes and mixed briefly. The samples were incubated for 30 minutes at 37°C, then each tube was mixed and 100 μ L added to LB-Kan plates and incubated overnight at 37°C. The number of colonies were counted on each plate and divided by the OD₆₀₀ for the respective strain.

Microscopy

For light microscopy, strains were grown overnight on LB agar supplemented with appropriate antibiotics and 0.1% arabinose. A small amount of bacteria was suspended in 5-7 μ L of 2 mM CaCl₂ on a microscope slide, covered with a cover slip, and viewed immediately for motility.

For electron microscopy, strains were grown to mid-log phase in LB supplemented with 100 μ g/mL streptomycin and 0.1% arabinose, then spun down and re-suspended in 0.15 M NaCl. Samples were adhered to carbon-formvar coated specimen grids (Electron Microscopy Sciences) and stained with 7% uranyl acetate for 7-10 seconds before viewing by microscopy.

Radio-labeled Palmitic Acid Incorporation Assay

Strains were grown in LB plus antibiotics at 37°C overnight and then normalized to an optical density of 0.4 at 600nm. Arabinose was added to a final concentration of 0.1% and the strains were incubated for five minutes before adding tritiated [³H] palmitic acid in ethanol to a final concentration of 25 μ Ci/mL. Cultures were grown at 37°C for 24 hours, 1mL samples were taken, spun down, the supernatant removed, and pellets re-suspended in 1X sample buffer and then boiled.

Samples were separated by SDS-15% polyacrylamide gel electrophoresis, and then fixed using a solution containing 5% glacial acetic acid, 5% isopropanol and water. The gel was then

treated with Autofluor (National Diagnostics) and imaged using Kodak X-Omat Blue XB-1 film at -80°C.

***In vivo* Colonization Assay**

The *in vivo* colonization assays were performed as described by Gardel and Mekalanos (1996) using 5-6 day old CD-1 suckling mice. Mixtures of wild-type strain KKV598 (O395 $\Delta lacZ$) with mutants KKV1965 ($\Delta flgP$), KKV2096 ($\Delta flgO$), KKV2062 ($\Delta flgP:flgP$), KKV2097 ($\Delta flgO:flgO$), KKV2147 ($\Delta flgP:flgP$ natural promoter), KKV2151 ($\Delta flgP:cj1026$), and KKV2152 ($\Delta flgP:flgP$ C18G natural promoter) were co-inoculated in a peroral inoculum ratio of approximately 10^5 mutant to 10^5 wild-type organisms. The small intestines were collected after 20-22 hours, homogenized, and the mutant to wild-type ratio was determined by dilution plating on LB agar containing X-Gal.

***In vitro* Competition Assay**

In vitro competition was carried out in 5mL of LB inoculated with mixtures of wild-type strain KKV598 (O395 $\Delta lacZ$) with mutants KKV1965 ($\Delta flgP$), KKV2096 ($\Delta flgO$), KKV2062 ($\Delta flgP:flgP$), KKV2097 ($\Delta flgO:flgO$), KKV2147 ($\Delta flgP:flgP$ natural promoter), KKV2151 ($\Delta flgP:cj1026$), and KKV2152 ($\Delta flgP:flgP$ C18G natural promoter) incubated at 37°C overnight. The mutant to wild-type ratio was determined by dilution plating on LB agar containing X-Gal.

RESULTS

***flgO* and *flgP* are regulated by the flagellar regulatory protein FlrC**

Microarray data collected previously suggested that *flgO* in *V. cholerae* is regulated by the Class III flagellar regulatory protein FlrC. Based on this information, we decided to test the promoter of *flgO* to confirm the findings seen in the microarray data. A plasmid containing the *flgOp* fused to *lacZ* was created (pKEK782) to observe differential expression of β -galactosidase activity in *V. cholerae* strains with deletions of the major flagellar regulatory genes. To observe which flagellar regulatory proteins influenced expression of the *flgO* promoter, the *flgOp-lacZ* fusion plasmid was put into a strain lacking each of the major regulators: FlrA, σ^{54} , FlrC, and σ^{28} . The results in Fig. 1 show that the promoter is active in the strain lacking σ^{28} (Δ *fliA*), but inactive in the absence of FlrC (Δ *fliC*). The flagellar regulators upstream of FlrC were also tested with results similar to the Δ *fliC* mutant strain (Fig.1). The data provides evidence that the *flgOp* is regulated by FlrC.

Bioinformatic predictions for FlgO and FlgP proteins

The *flgO* gene is predicted to encode a precursor protein of 222 amino acids with a molecular weight of 25 kDa. A first iteration PSI-BLAST search using FlgO indicated it contains a conserved domain within amino acids 52-218, COG5616, a predicted integral membrane protein of unknown function (Altschul *et. al.*, 1997; Schaffer *et. al.*, 2001). The PSI-BLAST was performed using the non-redundant database with an expect value of 10, and the BLOSUM62 matrix. Analysis of the protein using SignalP 3.0 neural network model provides three values to indicate a signal peptide (Bendtsen *et. al.*, 2004; Nielsen *et. al.*, 1997). The neural network model analysis did not provide a consensus to definitively indicate the presence

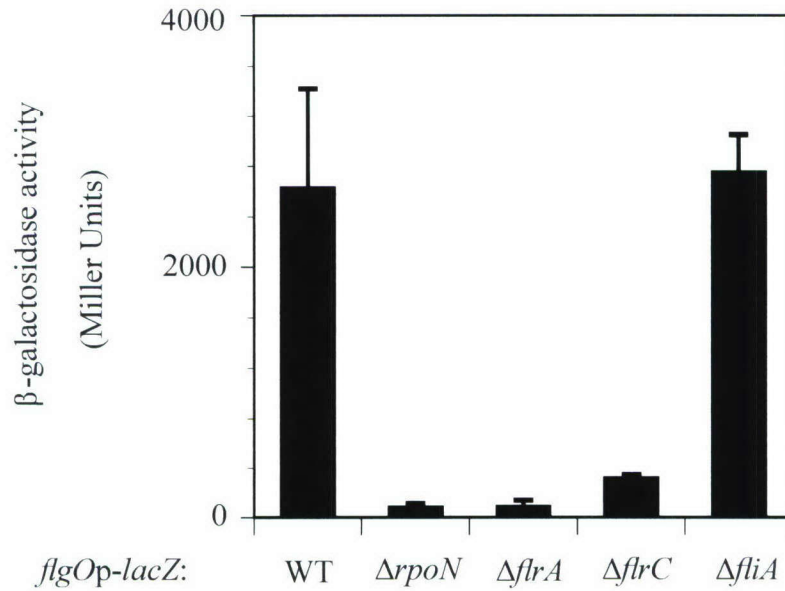


Figure 1. β-Galactosidase activity of the *flgO* promoter-*lacZ* transcriptional fusion in *V. cholerae*. The flagellar regulator FlrC is required for transcriptional activation of the *flgO* promoter. The *flgOp* was transcriptionally fused to *lacZ* in pRS551 (pKEK782) and transformed into wild-type (KKV598), *ΔrpoN* (KKV56), *ΔflrA* (KKV59), *ΔflrC* (KKV98), and *ΔfliA* (KKV1113) strains of *V. cholerae*. The results are the average of three replicate samples reported in Miller units. The cells were measured for β-galactosidase activity during logarithmic growth in LB.

of a signal peptide. However, the results suggested that it was likely that the protein contained an N-terminal signal peptide cleavage site and that it would be cleaved between amino acids 30 and 31. Similar results were obtained when the SignalP hidden Markov model analysis was used (Bendtsen *et al.*, 2004; Nielsen and Krogh, 1998; Nielsen *et al.*, 1997). When the protein was analyzed using Lipop 1.0 to see if it contained a signal peptidase II cleavage site (indicative of a lipoprotein) the results indicated that *flgO* was not a lipoprotein and it best matched the prediction for having transmembrane helices (Juncker *et al.*, 2003). The PSI-BLAST search also indicated that FlgO is encoded by other *Vibrio* species and *Photobacterium profundum* which are all in the family *Vibrionaceae*. The homologous proteins in *V. vulnificus* (CMCP6), *V. parahaemolyticus* (RIMD 2210633), *V. splendidus* (12B01), *V. fischeri* (ES114), *V. alginolyticus* (12G01), *V. angustum* (S14) and *Photobacterium profundum* (SS9, 3TCK and SKA34) share 56-86% identity and 77-96% similarity with FlgO in *V. cholerae*. In addition, when a Neighbor-Joining distance tree, using the Kimura method (pairwise alignment not a multiple alignment), is constructed using the PSI-BLAST results with a maximum distance difference of 0.75, the matches to the *V. cholerae* FlgO protein are all in the class gammaproteobacter (Altschul *et al.*, 1997; Schaffer *et al.*, 2001).

The *flgP* gene is predicted to encode a precursor protein of 145 amino acids with a molecular weight of 16kDa. A first iteration PSI-BLAST search using FlgP indicated it contains a conserved domain within amino acids 33-132, DUF400, a protein of unknown function that is also found in the pathogenic bacteria *Helicobacter pylori* and *Campylobacter jejuni* (Altschul *et al.*, 1997; Schaffer *et al.*, 2001). The PSI-BLAST was performed using the non-redundant database with an expect value of 10, and the BLOSUM62 matrix. Analysis of the protein using SignalP 3.0 neural network model provides three values to indicate a signal peptide. The analysis did not provide a consensus to definitively indicate the presence of a signal peptide.

However, the results suggested that it was likely that the protein contained an N-terminal signal peptide cleavage site. The hidden Markov model strongly supported that FlgP was a signal peptide with a probability of 0.999, while the cleavage position indicated by the model had a probability of 0.992 for the cleavage site to be between amino acid 23 and 24 (Bendtsen *et. al.*, 2004; Nielsen and Krogh 1998; Nielsen *et. al.*, 1997). When the protein was analyzed using LipoP 1.0 the results indicated that *flgP* contained a lipoprotein signal peptide and that the cleavage site was between amino acid 17 and 18 with the +2 amino acid position occupied by a glutamine (Juncker *et. al.*, 2003). The homologous proteins in *V. splendidus* (12B01), *V. vulnificus* (YJ016, CMP6), *V. parahemolyticus* (RIMD 2210633), *V. fischeri* (ES114), *V. alginolyticus* (12G01), *V. angustum* (S14) and *Photobacterium profundum* (SS9, 3TCK and SKA34) share 61-83% identity and 80-94% similarity with FlgP in *V. cholerae*. A protein in *Idiomarina baltica* contains 45% identity and 60% homology with 7% gaps. Homology with FlgP in other organisms is primarily within the DUF400 region. The other pathogenic bacteria that contain the DUF400 domain, *Helicobacter* and *Campylobacter* shared homology only in the DUF400 domain. In addition, when a Neighbor-Joining distance tree, using the Kimura method (pairwise alignment not a multiple alignment), is constructed using the PSI-BLAST results with a maximum distance difference of 0.75, the matches indicated that the *V. cholerae* FlgP protein has homology across several classes of bacteria that include eubacteria, alpha-, gamma-, and epsilonproteobacteria (Altschul *et. al.*, 1997; Schaffer *et. al.*, 2001).

Effect of *flgO* and *flgP* deletions on *V. cholerae* motility

Motility of the $\Delta flgO$ and $\Delta flgP$ mutant strains were measured in motility agar plates (Fig. 2). The swimming pattern observed for both strains showed non-motile phenotypes. Complementation of the $\Delta flgO$ mutant with pBAD24 containing the entire *flgO* gene (pKEK846

Figure 2. Motility measured by swarm assay on 0.3% semi-solid LB agar media

supplemented with 0.1% arabinose. (A) Strains: WT, wild-type (KKV598); $\Delta flgO$, non-motile (KKV2096); pKEK846, non-motile $\Delta flgO/pflgO$ (in KKV2096); pKEK978, non-motile $\Delta flgO/pflgO$ natural promoter (in KKV2096); pKEK1081, non-motile $\Delta flgO/pflgO-flgP$ (in KKV2096). (B) Strains: WT, wild-type (KKV598); $\Delta flgO$, non-motile (KKV2096); pKEK945, non-motile $\Delta flgO/pflgO$ -FLAG tag (in KKV2096); pKEK845, non-motile $\Delta flgO/pflgP$ (in KKV2096). (C) Strains: WT, wild-type (KKV598); $\Delta flgP$, non-motile (KKV1965); pKEK1044, $\Delta flgP/pflgP$ natural promoter (in KKV1965); pKEK1080, $\Delta flgP/pflgP$ C18G natural promoter (in KKV1965). (D) Strains: WT, wild-type (KKV598); $\Delta flgP$, non-motile (KKV1965); pKEK845, $\Delta flgP/pflgP$ (in KKV1965); pKEK944, decreased motility $\Delta flgP/pflgP$ -FLAG tag (in KKV1965); pKEK1079, non-motile $\Delta flgP/CJ1026$ (in KKV1965). (E) Strains: WT, wild-type (KKV598); $\Delta flgP$, non-motile (KKV1965); pKEK1037, $\Delta flgP/pflgP$ C18G (in KKV1965); pKEK1038, non-motile $\Delta flgP/pflgP$ -FLAG tag C18G (in KKV1965); pKEK1081, decreased motility $\Delta flgP/pflgO-flgP$ (in KKV1965).

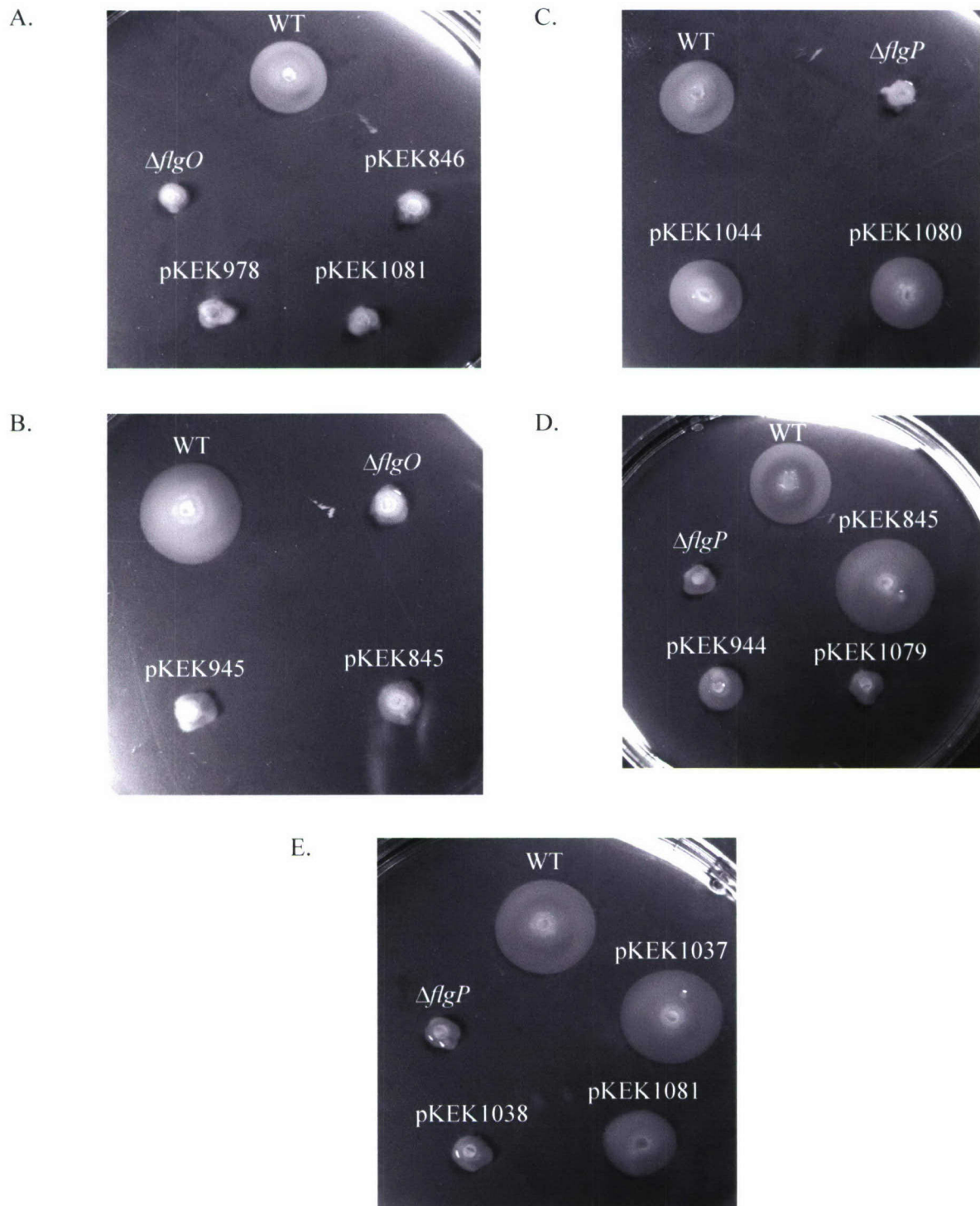


Figure 2

[Fig. 2A]) and the FLAG-tagged version (pKEK945 [Fig. 2B]) did not result in restoration of the wild-type phenotype. Additional plasmids were used to attempt restoration of motility to $\Delta flgO$ mutant; they included pBAD24 expressing FlgP (pKEK845 [Fig. 2B]), pBAD24 expressing FlgO and FlgP (pKEK1081 [Fig. 2A]), and pWSK30 expressing FlgO under its natural promoter's control (pKEK978 [Fig. 2A]).

Complementation of the $\Delta flgP$ mutant with pBAD24 containing the entire *flgP* gene (pKEK845 [Fig. 2D]) and a FLAG-tagged version (pKEK944 [Fig. 2D]) restored motility. However, the FLAG-tagged version was less motile than the wild-type. A pBAD24 plasmid containing *flgO* and *flgP* (pKEK1081 [Fig. 2E]) was used to attempt complementation of the $\Delta flgP$ mutant and was able to restore partial motility to the $\Delta flgP$ mutant. Additionally, the plasmid pKEK1079 containing a gene with homology in the DUF400 region to FlgP from *Campylobacter jejuni* 81-176, cj1026, was unable to restore motility to the non-motile $\Delta flgP$ mutant (Fig. 2D).

The motility phenotype also was confirmed using light microscopy. The $\Delta flgO$ and $\Delta flgP$ non-motile strains both contained small numbers of motile bacteria, but the vast majority of bacteria were non-motile when compared to the wild-type strain. Complementation of the $\Delta flgO$ mutant with pBAD24 containing the *flgO* gene produced similar results to the $\Delta flgO$ mutant with the vast majority of the bacteria observed remaining non-motile. Complementation of the $\Delta flgP$ mutant with pBAD24 containing the *flgP* gene resulted in wild-type levels of motile bacteria.

We used electron microscopy to directly observe cells of wild-type cholera (KKV598), $\Delta flgO$ (KKV2096), $\Delta flgO/pflgO$, $\Delta flgP$ (KKV1965), $\Delta flgP/pflgP$, and $\Delta flgP/pflgP$ C18G (Fig. 3). When the wild-type was compared to the $\Delta flgO$ mutant we observed that the mutants are primarily non-flagellated. However, when flagella are present they rarely have the natural curve

Figure 3. Transmission electron microscopy of *V. cholerae* $\Delta flgO$ and $\Delta flgP$ mutants. *V. cholerae* strains were grown to logarithmic phase, re-suspended in 0.15 M NaCl, then spread on carbon-formvar coated grids and stained with 7% uranyl acetate. (A and B) KKV598 (wild-type), (C and D) KKV2096 ($\Delta flgO$), (E and F) $\Delta flgO/pflgO$ (KKV2096 with pKEK846), (G and H) $\Delta flgP$ (KKV1965), (I and J) $\Delta flgP/pflgP$ (KKV1965 with pKEK845), (K and L) $\Delta flgP/pflgP$ C18G (KKV1965 with pKEK1037), (M and N) $\Delta flgP/pflgP$ natural promoter (KKV1965 with pKEK1044), (O and P) $\Delta flgP/pflgP$ C18G natural promoter (KKV1965 with pKEK1080). The bar in A, C, D, E, F, G, I, K, M, and O represent 500 nm while the bar in B, H, J, L, N, and P represent 2 microns.

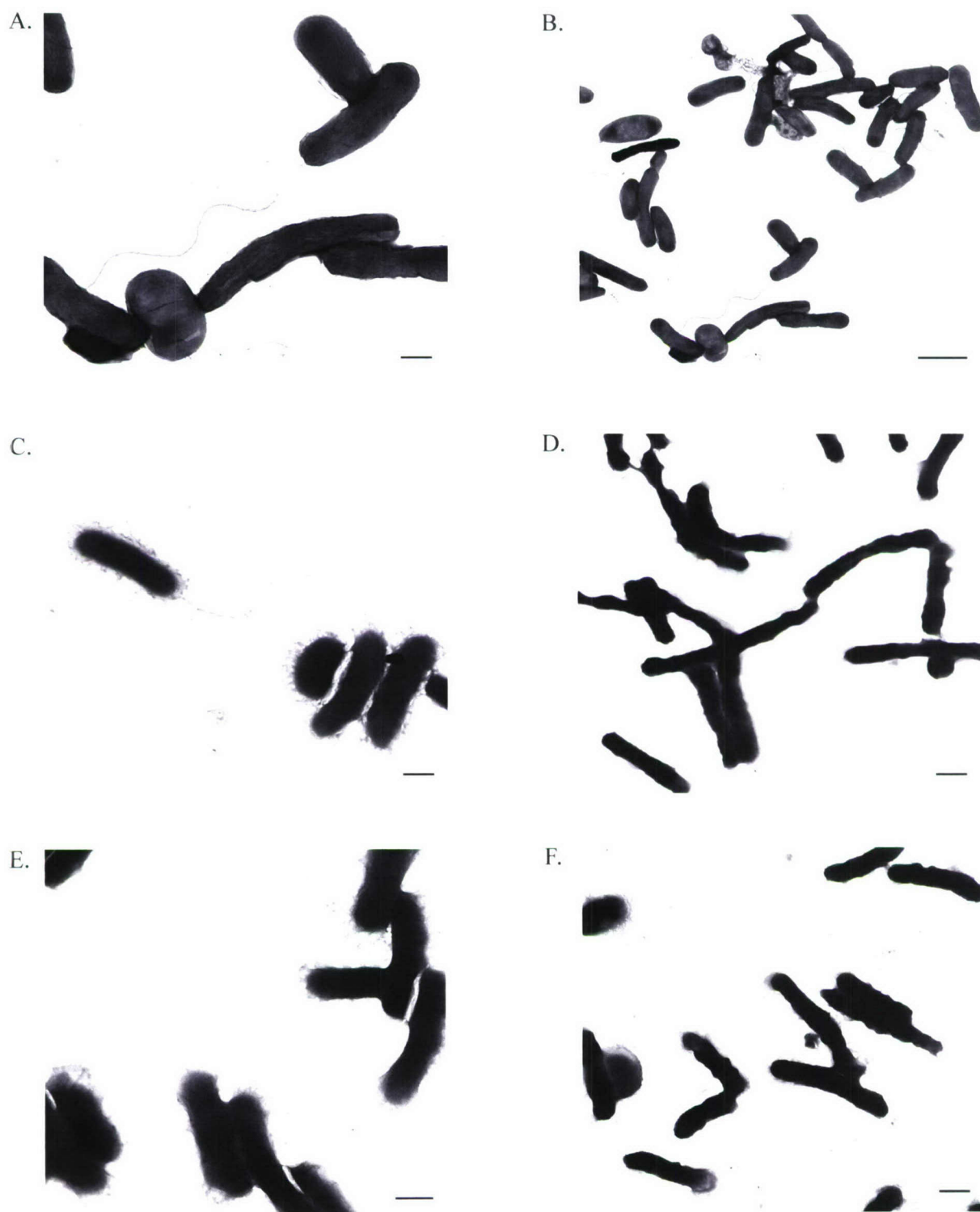
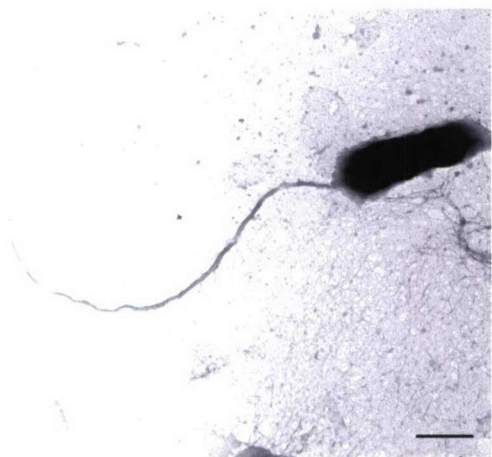


Figure 3

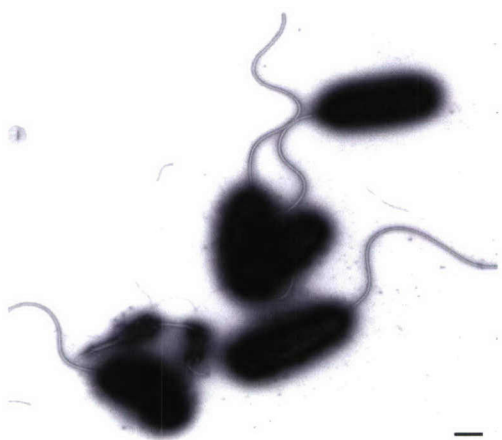
G.



H.



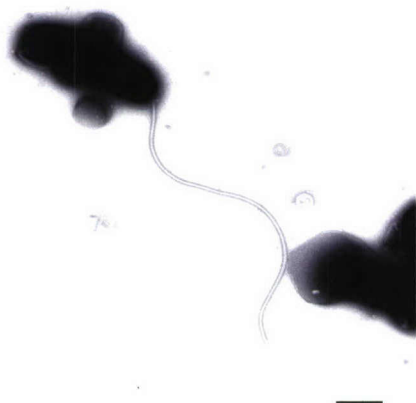
I.



J.



K.



L.

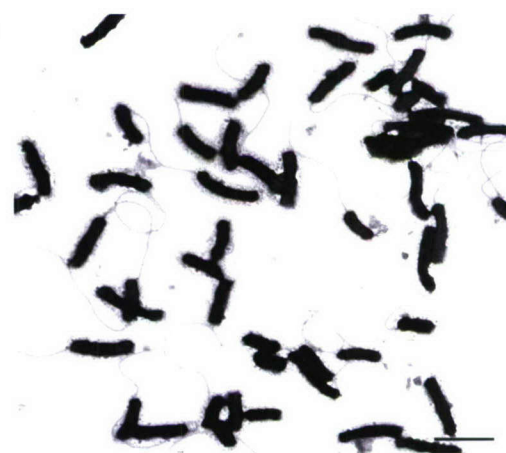
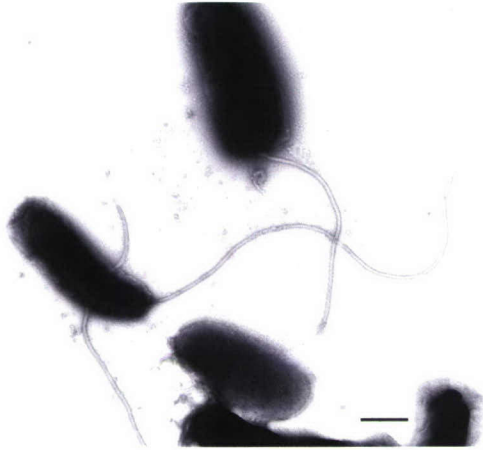


Figure 3 (cont.)

M.



N.



O.



P.

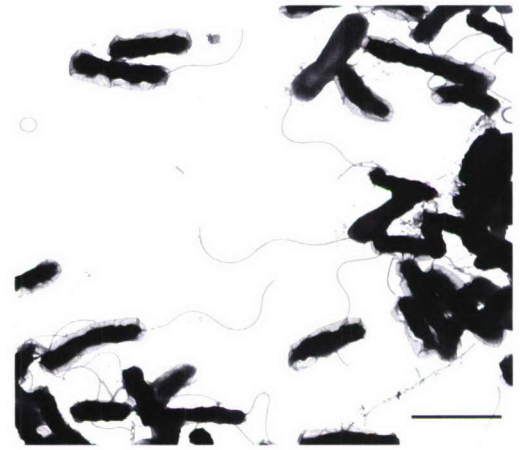


Figure 3 (cont.)

seen in the wild-type strain and the cell body length appears longer than wild-type, as if the cells are no longer separating at the appropriate time (Fig. 3A-D). When the $\Delta flgO$ mutant is complemented with pKEK846 there appears to be no significant change in the phenotype exhibited (Fig. 3E and F). When the $\Delta flgP$ mutant was examined, we observed that the cells were primarily flagellated but the flagella appeared fragile and many were broken; and in the wild-type flagella the edges of the flagella appear smooth, while in the $\Delta flgP$ mutant the edges are rough and discontinuous (Fig. 3G and H). The complemented $\Delta flgP/pflgP$ (pKEK845) strain exhibited wild-type flagellar structure (Fig. 3I). While the $\Delta flgP$ mutant complemented with *flgP* C18G (pKEK1037) restored the smooth edges to the flagella (Fig. 3K and L). We also observed the flagellar structure when the $\Delta flgP$ mutant was complemented with FlgP and FlgP C18G under the control of the natural promoter (pKEK1044 and 1080 respectively). The flagellar structures observed had the same characteristics as observed in the mutants with inducible plasmids (pKEK845 and pKEK1037) respectively (Fig. 3M-P).

Effect of *flgO* and *flgP* deletions on *V. cholerae* growth rate

A growth rate experiment was performed with the $\Delta flgO$ and $\Delta flgP$ mutants to determine if the deletion of either gene had a detrimental effect on the growth rate of *V. cholerae* in a rich liquid media (LB). A wild-type (KKV598) strain and the two mutant strains were grown at 37°C in a shaker for 7.5 hours with OD₆₀₀ measurements taken every hour up to 6 hours and the last measurement taken at 7.5 hours. Fig. 4 shows that both the $\Delta flgO$ and $\Delta flgP$ mutants exhibit wild type growth rates.

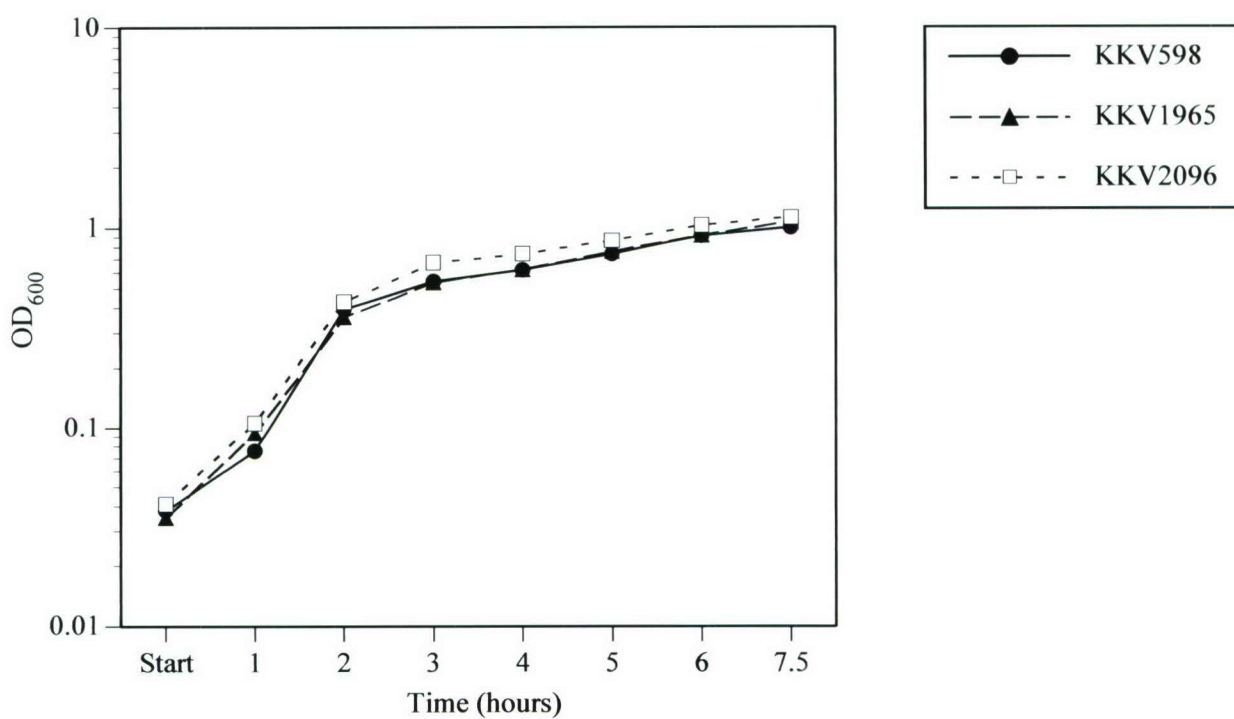


Figure 4. Growth rate for $\Delta flgO$ and $\Delta flgP$ mutants. Wild-type strain KKV598 (\bullet), $\Delta flgO$ strain KKV2096 (\square) and $\Delta flgP$ strain KKV1965 (\blacktriangle). Strains were grown in LB media at 37°C; growth rate was monitored by measuring OD_{600} .

Effect of *flgO* and *flgP* deletions on virulence factor expression

To determine if the absence of $\Delta flgO$ or $\Delta flgP$ has an effect on in vitro virulence factor expression, the mutant strains were grown under laboratory inducing conditions and then assayed for CT and TCP expression. CT was measured by GM₁-ELISA assay to detect CT in the supernatant and TCP was measured by CTX ϕ transduction. The positive control KKV598 was used as the baseline for the percent wild-type for both CT and TCP expression levels and KKV61 was used as the negative control for TCP expression. The $\Delta flgO$ mutant secreted 170.6% of wild-type levels of CT into the supernatant and expressed 190% of wild-type levels of TCP. The $\Delta flgP$ mutant secreted 164.2% of wild-type levels of CT into the supernatant and expressed 137.6% of wild-type levels of TCP (Table 4).

We also checked for CT and TCP expression in complemented $\Delta flgO$ and $\Delta flgP$ mutants (Table 4). Complementation of $\Delta flgO$ with pKEK846 ($\Delta flgO/pflgO$) resulted in a similar increase in CT expression while TCP expression was elevated to 446% of wild-type. When $\Delta flgO$ was complemented with pKEK978 ($\Delta flgO/pflgO$ natural promoter) the levels of CT were 214.7% of the wild-type and TCP was 317% of the wild-type. Complementation of $\Delta flgO$ with pKEK1081 ($\Delta flgO/pflgO-flgP$) resulted in CT levels 229.8% above wild-type and the highest TCP levels observed for both mutant strains of over 600% wild-type. None of the plasmids used to complement the $\Delta flgO$ mutant restored a motile phenotype to the mutant.

Complementation of $\Delta flgP$ with pKEK845 ($\Delta flgP/pflgP$ [Table 4]) resulted in a similar increase in CT expression while TCP expression was elevated to 313% of wild-type; even though the bacteria expressed a motile phenotype. Contrary to the results gathered with the inducible plasmid pKEK845, when pKEK1044 ($\Delta flgP/pflgP$ natural promoter [Table 4]) was used CT expression was seen to be 92.5% of wild-type, but TCP expression remained high at

Table 4. CT and TCP expression levels as percent wild-type for $\Delta flgO$ and $\Delta flgP$ mutants.

Strain Name	CT (%WT)	TCP (%WT)	Motility (+/-)
KKV598 (+)	100%	100.0%	+
KKV61 (-)	No Data	0.1%	No Data
$\Delta flgP$ (KKV1965)	164.2%	137.6%	-
$\Delta flgP/pflgP$ (pKEK845)	172.4%	313.3%	+
$\Delta flgP/pflgP$ natural promoter (pKEK1044)	92.5%	251.8%	+
$\Delta flgP/pflgP$ C18G (pKEK1037)	249.1%	246.4%	+
$\Delta flgP/pflgP$ C18G natural promoter (pKEK1080)	188.7%	171.6%	+
$\Delta flgP/pCJ1026$ (pKEK1079)	No Data	592.8%	-
$\Delta flgP/pflgO-flgP$ (pKEK1081)	143.3%	421.6%	+
$\Delta flgO$ (KV2096)	170.6%	190.0%	-
$\Delta flgO/pflgO$ (pKEK846)	185.0%	445.7%	-
$\Delta flgO/pflgO$ natural promoter (pKEK978)	214.7%	317.3%	-
$\Delta flgO/pflgO-flgP$ (pKEK1081)	229.8%	600.1%	-

251.81% wild-type. Complementation with both pKEK1037 and pKEK1080 ($\Delta flgP/pflgP$ C18G and $\Delta flgP/p\Delta flgO-flgP$ C18G [Table 4]) resulted in elevated levels of CT (249.1% and 188.7% respectively) and TCP (246.41% and 171.6 2% respectively) expression. The $\Delta flgP$ mutant was complemented with pKEK1081 ($\Delta flgP/pflgO-flgP$ [Table 4]) and resulted in increased levels of CT expression (143.3% wild-type) and the second highest levels of TCP expression for a $\Delta flgP$ mutant (421.58%), yet this strain had a motile phenotype. The plasmid containing the gene with homology to *flgP* from *C. jejuni*, pKEK1079 ($\Delta flgP/pcj1026$ [Table 4]) was assayed for TCP expression levels and resulted in the second highest levels of TCP observed for the mutants (592.8%).

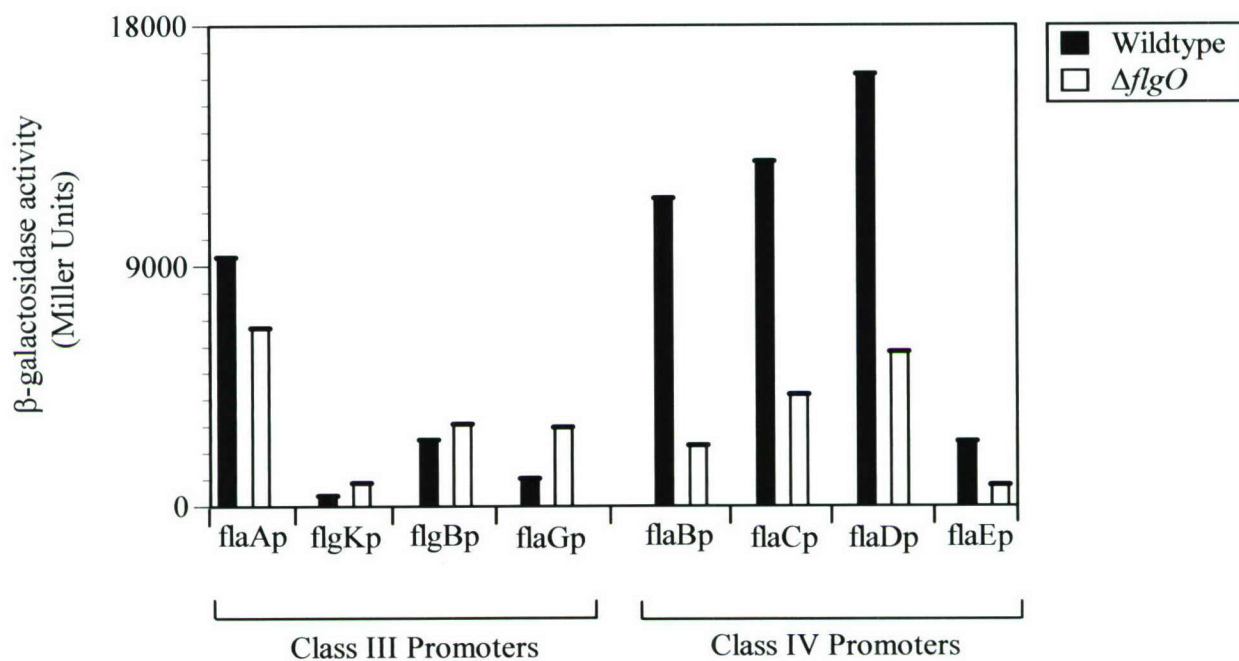
FlgO has a repressive effect on *Vibrio cholerae* Class IV flagellar gene expression

Since the *flgO* promoter appears to be σ^{54} and FlrC regulated, we decided to check the transcriptional activity of some of the Class IV gene promoters to determine the effect of the $\Delta flgO$ and $\Delta flgP$ mutations on their transcription. Transcription of four Class IV gene promoters was measured by promoter-*lacZ* transcriptional fusion plasmids (Klose and Mekalanos, 1998b) in wild-type, $\Delta flgO$ and $\Delta flgP$ strains (Fig. 5). We used promoters for *flaB*, *flaC*, *flaD*, and *flaE*, which are all σ^{28} dependent promoters, to check the Class IV gene expression levels (Prouty *et al.*, 2001). For the $\Delta flgO$ mutant we observed greater than 2-fold reductions for all of the Class IV promoters and up to a 5-fold reduction in *flaB* transcription (Fig. 5A).

Since the $\Delta flgO$ mutant expressed such low levels of the Class IV genes, we decided to determine at what point the gene expression returned to wild-type levels by checking for four Class III genes. The *flaA*, *flgK*, *flgB* and *flaG* promoters are all Class III promoters that are regulated by σ^{54} and FlrC (Prouty *et al* 2001). The results showed that transcription levels

Figure 5. β -Galactosidase activity of Class III and Class IV promoter-*lacZ* transcriptional fusions in *V. cholerae* FlgO and FlgP mutants. The *flaB*, *flaC*, *flaD* and *flaE* promoters are σ^{28} -dependent, as well as σ^{54} -, FlrA- and FlrC- dependent in *V. cholerae* (Class IV Promoters). The *flaA*, *flgK*, *flgB* and *flaG* promoters are FlrC-dependent, as well as σ^{54} -, and FlrA- dependent in *V. cholerae* (Class III Promoters) (Prouty *et. al.*, 2001). (A) *V. cholerae* strains KKV598 (wild-type), KKV2096 (Δ *flgO*) and carrying plasmids pKEK80 (*flaAp-lacZ*), pKEK79 (*flaBp-lacZ*), pKEK76 (*flaCp-lacZ*), pKEK77 (*flaDp-lacZ*), pKEK81 (*flaEp-lacZ*), pKEK331 (*flgKp-lacZ*), pKEK332 (*flgBp-lacZ*) and pKEK415 (*flaGp-lacZ*) were measured for β -Galactosidase activity during logarithmic growth in LB (Klose and Mekalanos, 1998a; Prouty *et. al.*, 2001). (B) *V. cholerae* strains KKV598 (wild-type), and KKV1965 (Δ *flgP*) carrying plasmids pKEK80 (*flaAp-lacZ*), pKEK79 (*flaBp-lacZ*), pKEK76 (*flaCp-lacZ*), pKEK77 (*flaDp-lacZ*), and pKEK81 (*flaEp-lacZ*) were measured for β -Galactosidase activity during logarithmic growth in LB (Klose and Mekalanos, 1998a). The results are the average of three replicate samples reported in Miller units.

A.



B.

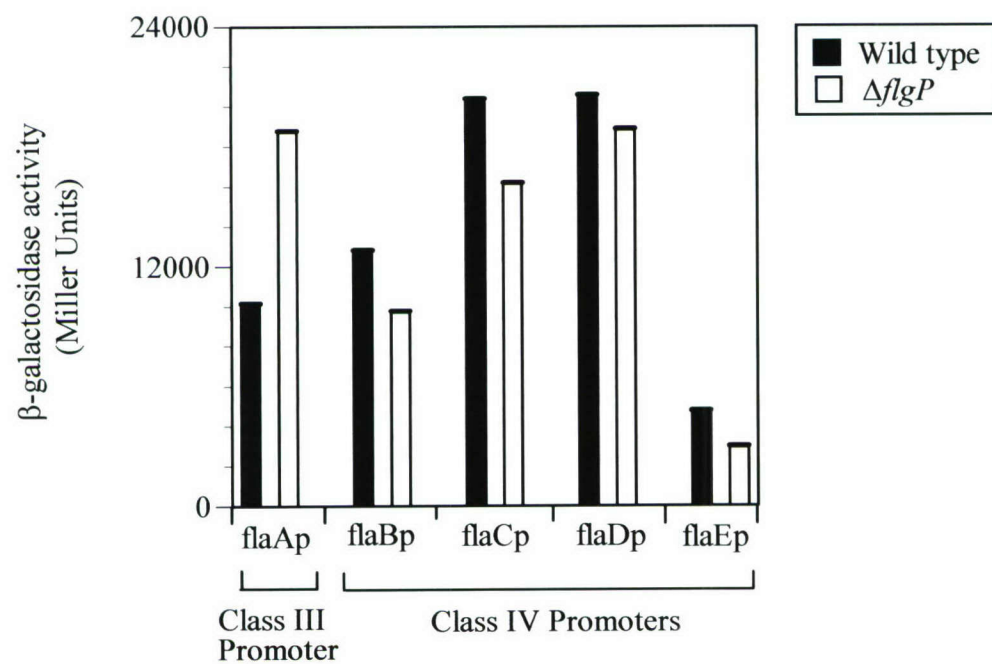


Figure 5

returned close to wild-type, or greater than wild-type levels. The *flaAp* activity showed just over a 1-fold decrease below wild-type levels, while *flgKp* showed a 2 fold increase, and *flaGp* showed almost a 3-fold increase in expression levels over wild-type. The Δ *flgP* mutant expression levels were near wild-type for all Class IV genes and nearly twice wild-type for *flaA*, a Class III gene (Fig. 5B).

***flgO* and *flgP* deletions prevent secretion of FlgM through the *V. cholerae* flagellum**

Based on the flagellar anomalies observed during electron microscopy and the decreased expression of Class IV genes in the Δ *flgO* mutant, we decided to perform an assay to check for secretion of the anti- σ^{28} factor FlgM. Correa *et. al.*, (2004) demonstrated that FLAG-FlgM is secreted through the flagellar export apparatus into the supernatant where it can be precipitated with TCA and assayed using SDS-PAGE and Western immunoblot with anti-FLAG antibodies (Sigma). We used the wild-type *V. cholerae* strain with the FLAG-*flgM* plasmid as a positive control and the Δ *fliF* *V. cholerae* strain as a negative control, from the experiment performed by Correa *et. al.*, (2004). The FLAG-*flgM* plasmid was then transformed into Δ *flgO* and Δ *flgP* and the assay was performed (Fig. 6).

FLAG-*flgM* could be detected in the cell pellet (P) of all strains tested (Fig. 6). However, FLAG-*flgM* was only detected in the supernatant (S) of the positive control wild-type strain, while FLAG-*flgM* could not be detected in the negative control (Δ *fliF*) or the supernatant of either Δ *flgO* or Δ *flgP* (Fig. 6). These results indicate that there may be some mechanism that is blocking secretion of FlgM through the flagella into the supernatant.

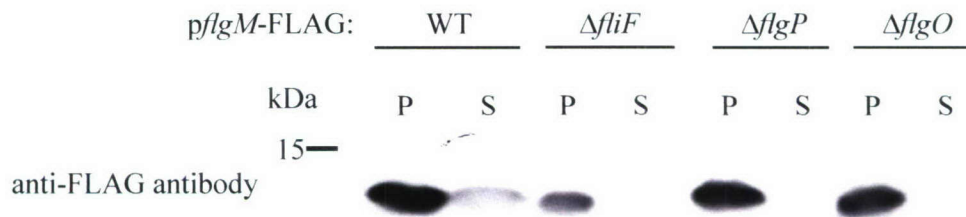


Figure 6. Detection of FLAG-FlgM in cell pellets and supernatants of Δ flgO and Δ flgP mutants. Correa *et. al.*, 2004 described a method to visualize FlgM secreted through the *V. cholerae* flagellum into the supernatant when labeled with a FLAG epitope. FLAG labeled FlgM was expressed from the plasmid pKEK474 (Correa *et. al.*, 2004) in wild-type (KKV598), Δ fliF (KKV2147), Δ flgO (KKV2096), and Δ flgP (KKV1965). Bacterial pellets (P) and supernatants (S) were visualized using Western Immunoblot analysis with anti-FLAG antibodies.

FlgO is a secreted protein and FlgP is an outer membrane lipoprotein

We tested the proteins for secretion out of the cytoplasm by constructing plasmids that contained *flgO-phoA* and *flgP-phoA* fusions (pKEK1036 and pKEK1035 respectively). The plasmids were transformed into $\Delta flgO$ and $\Delta flgP$ mutants respectively, and then grown in the presence of 0.1% arabinose. We then assayed for alkaline phosphatase activity, which is indicative of secretion out of the cytoplasm (Michaelis, 1983). We observed that both proteins appear to be secreted; *flgO* shows a much higher level of secretion than *flgP* (Fig. 7). KKV2095 was used as a positive control strain and the negative control strains for $\Delta flgO/pflgO-phoA$ and $\Delta flgP/pflgP-phoA$ contained the pBAD24 plasmid without the gene-*phoA* fusion (Fig. 7).

Next we checked for localization of FlgO and FlgP to the outer membrane of *V. cholerae*. This was determined using the FLAG-tagged FlgO and FlgP proteins. The outer membrane protein enrichment assay described by Lohia *et. al.*, (1984) was modified for *V. cholerae* to isolate the outer membranes for probing using anti-FLAG M2 antibody (Fig. 8A). To ensure that comparable amounts of protein were loaded, a coomassie stained gel was used and the 37kDa protein was the standard (Fig. 8B). The results indicated that FlgO does not appear to localize to the outer membrane extract (Fig. 8A). In contrast, FlgP was present in high quantities in the outer membrane extract. However, when cysteine¹⁸ is mutated to glycine there is a significant decrease in the amount of FlgP that localizes to the outer membrane extract (Fig. 8A).

Localization to the outer membrane suggests that FlgP may be a lipoprotein, but to confirm this incorporation of a labeled lipid was performed. Incorporation of the labeled lipid was accomplished by growing wild-type bacteria and mutant strains, $\Delta flgP$, $\Delta flgP/pflgP$ -FLAG, and $\Delta flgP/pflgP$ C18G-FLAG for 24 hours in LB with appropriate antibiotic with 0.1% arabinose in the presence of H³ palmitic acid. Fig. 8C shows that $\Delta flgP/pflgP$ -FLAG incorporated the H³ palmitic acid, while $\Delta flgP$ and $\Delta flgP/pflgP$ C18G-FLAG did not incorporate the H³ palmitic acid

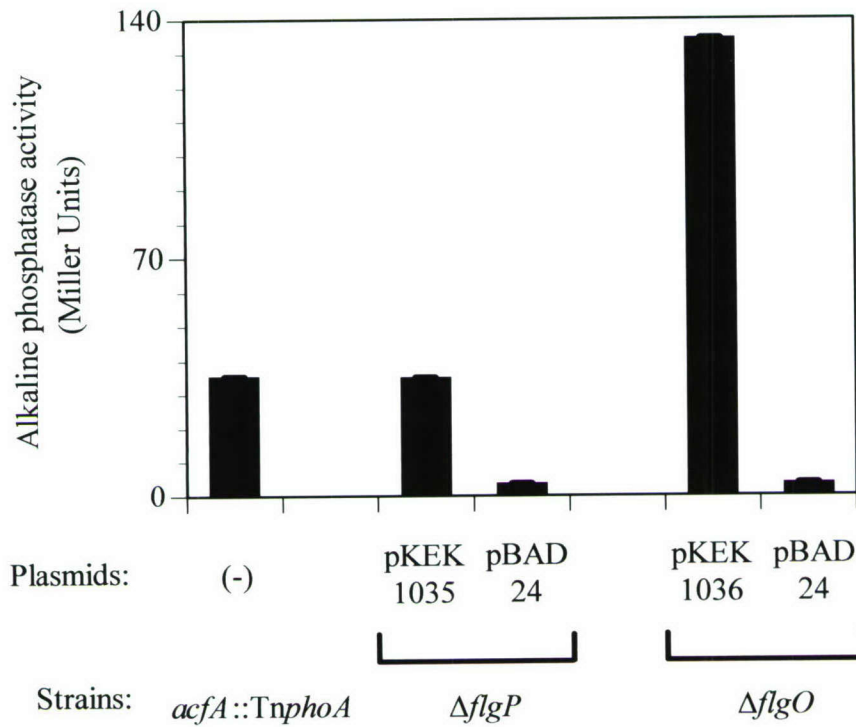


Figure 7. Alkaline phosphatase activity of *flgO-phoA* and *flgP-phoA* translational fusions in *V. cholerae*. *flgO* and *flgP* were fused to *E. coli phoA* in pBAD24 (pKEK1036 and pKEK1035 respectively) and transformed into $\Delta flgO$ (KKV2096) and $\Delta flgP$ (KKV1965) *V. cholerae* strains respectively. pBAD24 was transformed into $\Delta flgO$ (KKV2096) and $\Delta flgP$ (KKV1965) *V. cholerae* strains as a negative control and *acfA::TnphoA* (KKV2095) was used as a positive control. The results are the average of three samples reported in Miller units. The cells were measured for alkaline phosphatase activity during logarithmic growth in LB.

Figure 8. Outer membrane localization and lipidation assay of FlgO and FlgP. FLAG

labeled FlgO and FlgP were expressed in the following *V. cholerae* strains: (-) (KKV1965 with pKEK845), FlgP-FLAG (KKV1965 with pKEK944), FlgP-FLAG C18G (KKV1965 with pKEK1038), and FlgO-FLAG (KKV2096 with pKEK945). (A) Bacterial pellets (WC) and enriched outer membrane preparation (OM) were visualized using Western Immunoblot analysis with anti-FLAG antibodies. (B) Bacterial pellets (WC) and enriched outer membrane preparation (OM) were stained with coomassie blue and the 37kDa protein was used to normalize protein concentration. (C) Visualization of lipid incorporation into FlgP was performed using *V. cholerae* strains: (-) KKV598 (wild-type), (-) $\Delta flgP$ (KKV1965), FlgP-FLAG (KKV1965 with pKEK944), and FlgP-FLAG C18G (KKV1965 with pKEK1038) that were grown in the presence of tritiated palmitic acid for 24 hours with antibiotics and 0.1% arabinose, run on 15% SDS-PAGE gel, and imaged using Kodak X-Omat Blue XB-1 film at -80°C.

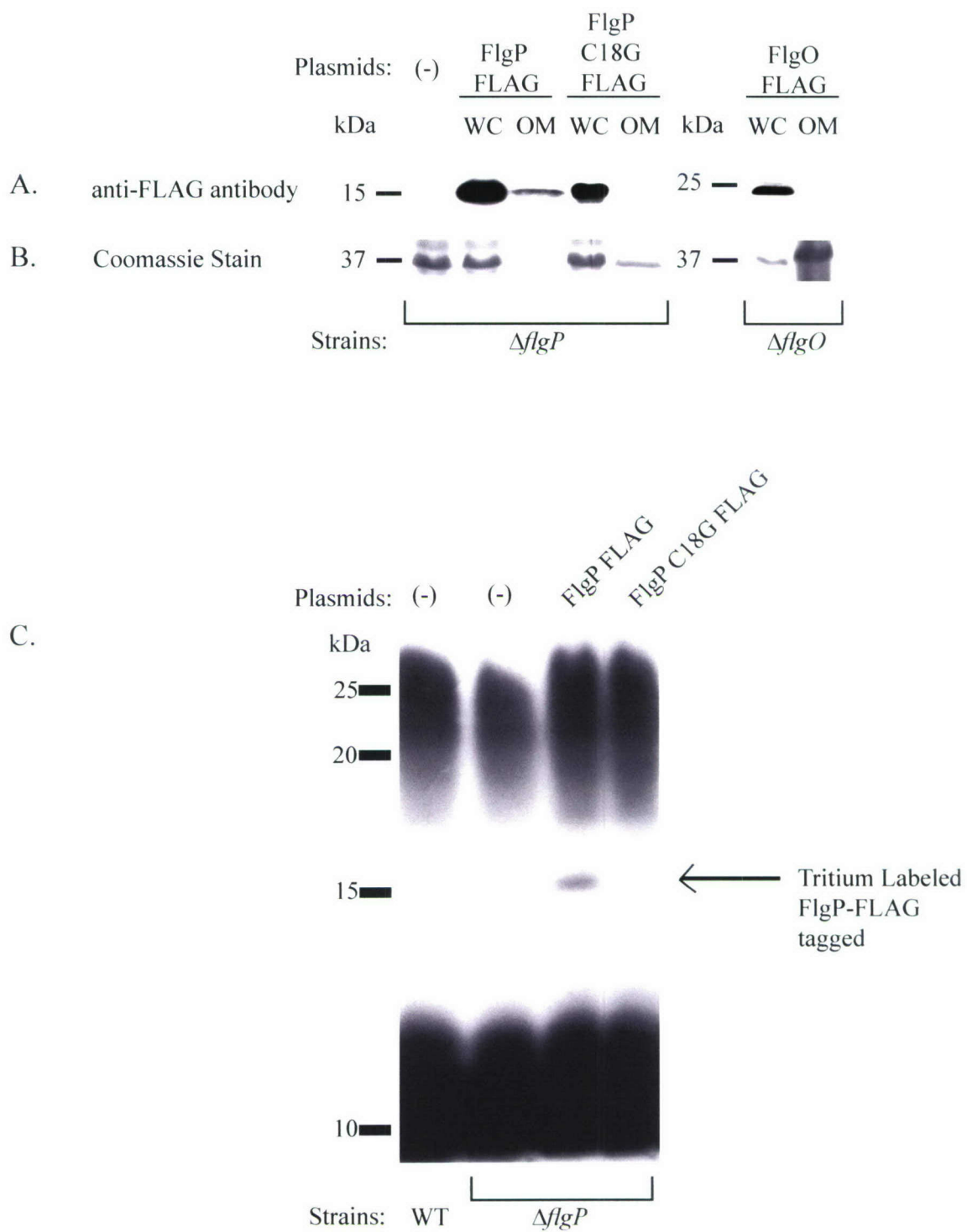


Figure 8

at detectable levels. A band does not appear in the wild-type, but this could be due to very low levels of FlgP expression (Fig. 8C). Incorporation of the H³ palmitic acid confirms that FlgP is a lipoprotein and that C18 is the site of lipidation as predicted by LipoP 1.0.

Colonization of the infant mouse intestine

We tested the ability of our mutants to colonize the suckling mouse small intestine (Fig.9). The *in vivo* competition experiments were performed with the following strains against a wild type strain: $\Delta flgO$, $\Delta flgO/pflgO$, $\Delta flgP$, $\Delta flgP/pflgP$, $\Delta flgP/pflgO.flgP$, $\Delta flgP/pflgO.flgP$ -C18G and $\Delta flgP$:CJ1026. The $\Delta flgO$ competitive index was only slightly lower than the wild-type, whereas, when $\Delta flgO$ was complemented, $\Delta flgO/pflgO$ (pKEK846), there was a greater decrease in the competitive index. The results indicate that *flgO* does not appear to have a significant effect on colonization, while expression of *flgO* from an inducible plasmid has a detrimental effect on colonization.

The competitive index for the $\Delta flgP$ strain showed a greater than 10-fold decrease in colonization compared to wild-type, while complementation with $\Delta flgP/pflgP$ (pKEK845) resulted in the competitive index dramatically improving to near wild-type levels. The strain $\Delta flgP$:CJ1026 (pKEK1079) showed a lower competitive index than the $\Delta flgP$ mutant. With the possibility that over-expression of FlgP, could potentially have a negative effect on *V. cholerae*, we tested $\Delta flgP/pflgO.flgP$ (pKEK1044) that has the *flgP* gene controlled by its natural promoter. We also tested $\Delta flgP/pflgO.flgP$ C18G (pKEK1080) that prevents lipidation of FlgP under its natural promoter. The strain $\Delta flgP/pflgO.flgP$ was able to colonize the infant mouse small intestine as well as wild-type, while $\Delta flgP/pflgO.flgP$ C18G showed a defect in

colonization comparable to the $\Delta flgP$ mutant. This suggests that lipidation of FlgP plays an important role in the ability of *V. cholerae* to colonize the infant mouse intestine.

Figure 9. Intestinal colonization of $\Delta flgO$ and $\Delta flgP$ mutants. The assay was performed as described in the text (Materials and Methods). *V. cholerae* strains $\Delta flgO$ (KKV2096), pKEK846 ($\Delta flgO/pflgO$), $\Delta flgP$ (KKV1965), pKEK845 ($\Delta flgP/pflgP$), pKEK1044 ($\Delta flgP/pflgP$ natural promoter), pKEK1080 ($\Delta flgP/pflgP$ C18G natural promoter), and pKEK1079 ($\Delta flgP/pCJ1026$) were co-inoculated with wild-type KKV598 (O395 $\Delta lacZ$) perorally into infant mice at a ratio of ~1:1. Homogenates of the mice small intestines were collected at 24 hours post inoculation and colony forming units were counted of the respective wild-type and mutant strain. The competitive index is the output ratio of mutant to wild-type divided by the input ratio of mutant to wild-type and each data point represent an individual mouse.

* Wild-type colonies were detectable but mutant colonies were below the detectable threshold. For these strains the competitive index was calculated using the actual number of wild-type colonies and one mutant colony.

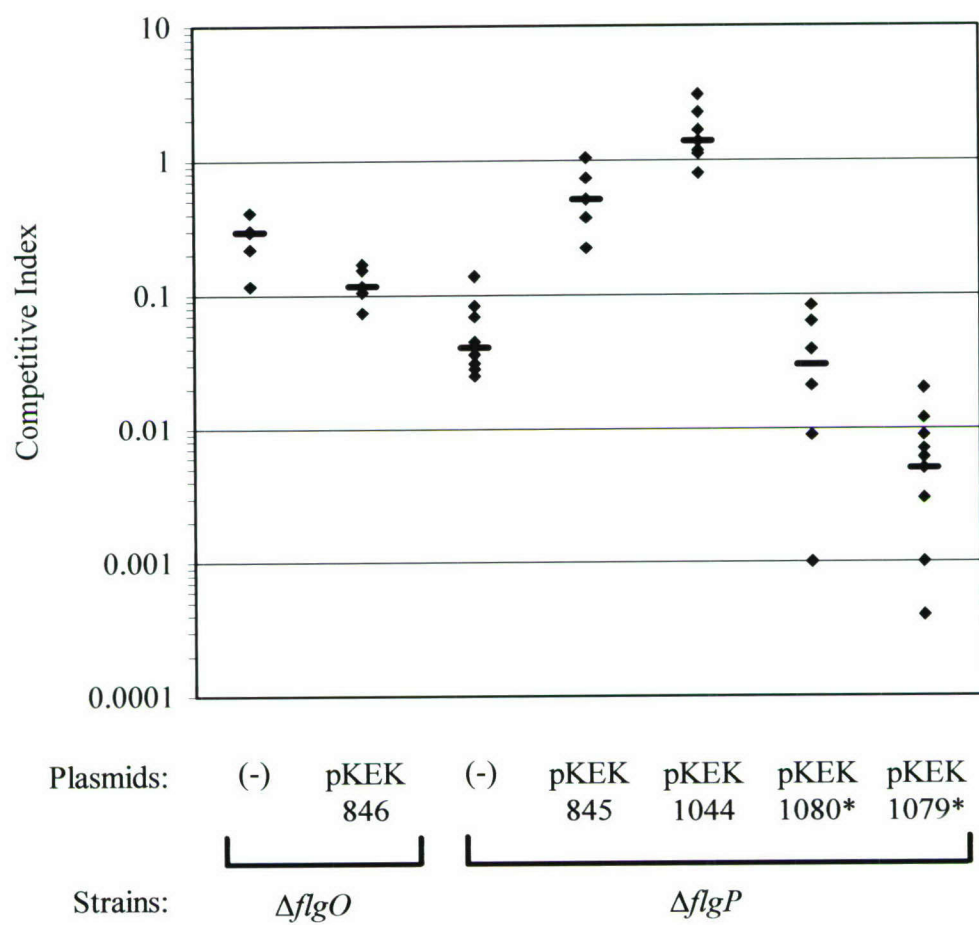


Figure 9

DISCUSSION

The genes VC2206 and VC2207 were discovered when Heidelberg *et. al.*, (2000) sequenced both chromosomes of the *V. cholerae* genome. VC2206 and VC2207 were both labeled as hypothetical proteins with unknown functions. Microarray analysis of KKV56 (O395 $\Delta rpoN1$, $\Delta lacZ$), KKV59 (O395 $\Delta flrA1$, $\Delta lacZ$), KKV98 (O395 $\Delta flrC1$, $\Delta lacZ$) and KKV1113 (O395 $\DeltafliA1$, $\Delta lacZ$) provided evidence that VC2206 and VC2207 were regulated by the Class III flagellar regulatory protein FlrC (Personal communication, Dr. Klose). Thus, this thesis project begins the characterization of VC2206 (*flgP*) and VC2207 (*flgO*). We chose to characterize these genes because the microarray evidence supported their regulation by flagellar regulatory mechanisms. This is of particular interest due to the fact that even though the exact relationship between flagellar synthesis, motility and virulence is not completely understood; it is clear that a relationship exists that may be exploitable for vaccine development. Guentzel and Berry (1975) showed that non-motile strains of Classical and El Tor biotype cholera showed reduced levels of virulence. Coster *et. al.*, (1995) created a non-motile strain with decreased virulence and increased immunogenicity as a live attenuated vaccine candidate.

Bioinformatic Analysis

The bioinformatic analysis of the FlgO and FlgP proteins provided predictions that helped to establish the process we used to analyze the proteins. Based on the predictions that both proteins had a high probability of containing signal peptides and the prediction that FlgP contained a signal peptidase II cleavage site for addition of a lipid; we hypothesized that FlgO was a secreted protein and FlgP was an outer membrane lipoprotein. Growth of the *V. cholerae* strains lacking FlgO or FlgP resulted in non-motile phenotypes in swarm agar. Based on the

observations that the *flgO* promoter is regulated by FlrC, that FlgO is a predicted secreted protein, that FlgP is a predicted lipoprotein, and the lack of FlgO or FlgP in *V. cholerae* results in a non-motile phenotype, we hypothesized that secretion of FlgO is required for motility and enhanced colonization, and that lipidation and localization of FlgP to the outer membrane is required for motility and enhanced colonization. Our objective was to characterize these two proteins and determine their roles in motility and virulence, providing evidence that one or both would make potential targets for vaccine development.

***flgO* and *flgP* are transcriptionally regulated by FlrC**

Initial studies confirmed results seen in the microarray data that *flgO* and *flgP* are regulated by FlrC. On the chromosome, *flgO* is transcribed immediately upstream of *flgP* and was thought to contain a promoter sequence that was recognized by FlrC. When we tested the *flgO* promoter in *V. cholerae* using an *flgO-lacZ* fusion (pKEK782), results supported those observed in the microarray data. Transcription of the *flgO* promoter was active in KKV1113 (Δ *fliA*), which regulates Class IV genes, but was inactive in the rest of the strains with deletions in Class I, II and III transcriptional regulators. Since the regulation is a cascade, interrupting the earlier regulators prevents activation of the downstream genes (Prouty *et. al.*, 2001). The location of *flgO* and *flgP* on the *V. cholerae* chromosome would put them in flagellar chromosome region I immediately adjacent to *flgN* and *flgM* as described by Prouty *et. al.*, (2001). They are located in the same region as genes that form the basal body rod, rings, and hook (*flgB-J*); the hook filament junctions (*flgK-L*); the filament (*flaA* and *flaC*); and chemotaxis genes (*cheV* and *cheR*) (Prouty *et.al.*, 2001). They are labeled according to the order they are transcribed, so on the chromosome *flgP* is immediately adjacent to *flgN*, while *flgO* is immediately adjacent to *flgP*.

FlgO is a secreted protein and FlgP is an outer membrane lipoprotein

We were able to provide evidence that both FlgO and FlgP are secreted proteins by creating gene-*phoA* translational fusions. Based on the results of our alkaline phosphatase assays (Fig. 7), both proteins were secreted at levels equal to or greater than the control strain (KKV2095) and this was not a result of the plasmid from which the genes were expressed.

Bioinformatic analysis of the predicted amino acid sequence of FlgP using LipoP 1.0 showed the presence of a 17 amino acid N-terminal sequence with the characteristics of a lipoprotein signal sequence specifically cleaved by signal peptidase II. Cleavage at amino acid 17 resulted in an exposed cysteine residue at the N-terminus of the mature protein that would be available for acylation. The second amino acid after the cleavage site can be used to predict where the lipidated protein will localize within the cell using what is called the +2 rule (Seydel *et. al.*, 1999). FlgP has a glutamine in the +2 position which, according to Seydel *et. al.*, (1999), predicts the lipoprotein will be sorted to the outer membrane. Seydel *et. al.*, (1999) tested the +2 rule for *E. coli*, which also is a gram negative bacterium and we expect that the rule should hold true for other gram negative bacteria such as *V. cholerae*.

Further experimentation with FLAG tagged versions of FlgO and FlgP showed that FlgO-FLAG did not localize to the outer the membrane, while FlgP-FLAG could be isolated from the outer membrane (Fig. 8A). This supported the prediction that FlgP was an outer membrane lipoprotein. We created a FLAG tagged FlgP protein and changed the cysteine at the predicted signal peptidase II cleavage site to a glycine. The C18G mutation would prevent cleavage and acylation of the FlgP protein and ultimately prevent localization to the outer membrane. It is clear that almost no FlgP C18G FLAG protein could be detected in the outer membrane of *V. cholerae* (Fig. 8A). While this observation provides compelling evidence that FlgP localizes to the outer membrane, it does not directly show that FlgP is lipidated. To confirm that FlgP was

lipidated, we used H³ palmitic acid to visualize the lipidation of FlgP with palmitic acid (Fig. 8C). The results support our initial hypothesis that FlgP was a lipoprotein that was located in the outer membrane.

FlgO and FlgP are required for motility

We created chromosomal deletions of *flgO* and *flgP* in KKV598 (wild-type) *V. cholerae* and tested them for motility. Our results indicate that complementation of $\Delta flgO$ with *pflgO* (KKV2096 with pKEK846) did not restore wild-type motility, while complementation of $\Delta flgP$ with *pflgP* (KKV1965 with pKEK845) restored wild-type motility to the mutant strain in swarm agar. These results were confirmed using light microscopy to observe the motility of the bacteria. Initially we thought that the $\Delta flgO$ mutation was polar on expression of the downstream *flgP* gene, but the deletion was sequenced and additional plasmids made to provide evidence that the $\Delta flgO$ mutation was non-polar. A plasmid that contained both *flgO* and *flgP* was created (pKEK1081) so that both proteins would be expressed (spatially and temporally relative to each other). Even if there was a polar deletion, this plasmid should complement the $\Delta flgO$ mutant. We found that this plasmid would not complement $\Delta flgO$. However, when put into $\Delta flgP$, restoration of a motile phenotype was observed. We do not believe that the $\Delta flgO$ mutation is a polar mutation. However, our inability to complement this mutant has led us to believe that FlgO must be present in a specific ratio to FlgP, or at a specific time during flagellar synthesis, and when we express the protein on a plasmid we are unable to reproduce those conditions necessary to achieve complementation.

The $\Delta flgP$ mutant was complemented with five additional plasmids containing variations of the *flgP* gene or a gene from *C. jejuni* with homology to FlgP. The only plasmids that did not complement the $\Delta flgP$ mutant were pKEK1038 (*flgP*-FLAG tag C18G in pBAD24) and

pKEK1079 (CJ1026 in pBAD24). Complementation with pKEK944 resulted in decreased motility; the addition of the FLAG tag appears to prevent the FlgP protein from acting in its wild-type capacity. This, in conjunction with the C18G mutation, may have prevented adequate amounts of the protein from localizing to the appropriate place within the cell. Each mutation individually, addition of the FLAG-tag and mutation of C18G, did not completely inhibit motility, but a compounding effect appears to prevent a motile phenotype. Complementation of $\Delta flgP$ mutant with pKEK1079 did not restore the motile phenotype. An explanation for this may be that in *C. jejuni* a secondary chaperone protein FlgQ is required to stabilize or localize the *C. jejuni* FlgP to the outer membrane (Sommerlad and Hendrixson, 2006). There is no homolog to FlgQ in *V. cholerae*, and we did not include this gene on our expression vector. Another possibility is that the amount of homology between the two proteins is not adequate to complement the deletion in *V. cholerae*.

We used TEM to visualize the flagella of the $\Delta flgO$ and $\Delta flgP$ mutants. Swarm assays with non-motile phenotypes did not tell us whether flagella were being produced or were just non-functional. Visualization allowed us to see that flagella were in fact being produced in the mutant strains. The $\Delta flgO$ mutant produces a flagella but the flagella lacks a smooth surface appearance like the wild-type (Fig. 3C). In addition, an extended body structure is noticeable for many of the bacteria viewed (Fig. 3D). Complementation of the $\Delta flgO$ mutant with pKEK846 (*pflgO*) does not restore a wild-type flagellar structure (Fig. 3E), and the majority of bacteria viewed did not have an attached flagella, nor were broken flagella apparent on the grid (Fig. 3F). The decreased expression of Class IV flagellins (Fig. 5A) may be the reason that few and or shortened (broken) flagella were seen. Klose and Mekalanos (1998b) created a *flaBCDE* mutant and observed a similar phenotype, where many of the bacteria had sheared or short flagella, and

they predicted it was due to fragile flagella that were damaged during sample preparation.

However, this does not explain the extended cell bodies observed in the $\Delta flgO$ mutant.

The $\Delta flgP$ mutant also produced fragile flagella. The images in Fig. 3G and H show the flagella having rough discontinuous edges and broken flagella can be seen on the grid. When the $\Delta flgP$ mutant was complemented with pKEK845 (*pflgP*) the flagella returned to wild-type structure, exhibiting clear smooth edges and nearly all flagella were attached to the cell body (Fig. 3I and J). We also complemented the $\Delta flgP$ mutant with pKEK1037 (*pflgP* C18G), resulting in near wild-type flagellar structure (Fig. 3K and L). This indicates that lipidation of FlgP is not required for the production of wild-type flagella. To ensure that overproduction of the FlgP protein was not affecting the observed results, images were also taken of a $\Delta flgP$ mutant with pKEK1044 (*pflgP* natural promoter) and a $\Delta flgP$ mutant with pKEK1080 (*pflgP* C18G natural promoter). The results observed confirmed that complementation with FlgP or FlgP C18G from an inducible plasmid or under the control of its natural promoter restore the wild-type flagellar structure phenotype.

FlgO and FlgP virulence factor expression and roles in enhanced intestinal colonization

The data collected from the CT and TCP assay showed increased expression of CT and TCP in all strains tested except $\Delta flgP/pflgP$. We did not see any major decreases in CT or TCP expression in the $\Delta flgP$ mutant (KKV1965) or the $\Delta flgP/pflgP$ C18G natural promoter (KKV1965 with pKEK1080) strains; even though, both of these strains showed greater than 10 fold decreases in the competitive index for colonization. Gardel and Mekalanos (1996) showed that non-motile mutants exhibited increased CT and TCP expression while few or no defects in intestinal colonization were identified. This provides some insight as to why we see the increased CT and TCP expression in the non-motile strains.

FlgP appears to play a role in intestinal colonization of the infant mouse. We observed a greater than 10-fold decrease in the competitive index of the $\Delta flgP$ mutant (KKV1965). When complemented with pKEK845 (*pflgP*) or pKEK1044 (*pflgP* natural promoter), the competitive index returned to near wild-type levels. We also complemented the $\Delta flgP$ mutant (KKV1965) with pKEK1080 (*pflgP* C18G natural promoter) and the competitive index showed a greater than 10 fold reduction without lipidation (Fig. 9). Interestingly the $\Delta flgP/pflgP$ C18G natural promoter (pKEK1080) strain was motile in swarm agar (Fig. 2C). This indicates that lipidation of FlgP is required for enhanced colonization, but is not required for motility.

Future Directions

With each new piece of data collected another piece of the FlgO and FlgP puzzle is made clear. However, each piece of data raises additional questions. We recently identified a suppressor mutant that arose from the $\Delta flgO$ mutant. We tried several ways to complement the $\Delta flgO$ mutant and were unsuccessful, yet the mutant was able to regain motility through spontaneous mutation. Identification of the mutation would provide valuable information on FlgO and its interactions with other cellular components. We have identified FlgP as an outer membrane lipoprotein that requires lipidation and localization to the outer membrane for enhanced colonization of the infant mouse small intestine but not motility. Is FlgP an adhesin that allows the bacteria to colonize the small intestine and if so, would it make a viable target for vaccine development? The answers to these questions and many others will be found and we hope that by characterizing FlgO and FlgP we have provided a solid starting point for continued research.

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VITA

David Morris was born in Victoria, Texas. After completing his high school education at Goliad High School, Goliad, Texas, he entered the United States Air Force Academy in Colorado Springs, Colorado. He received the degree of Bachelor of Science in Biology from the United States Air Force Academy. After graduation he married his wonderful wife. He entered active duty military service and served three years before entering the Graduate School at the University of Texas at San Antonio to complete a Masters in Biology. Upon arrival at UTSA he started his graduate thesis research under the mentorship of Dr. Karl Klose.